

TRYPANOSOMA CONGOLENSIS: CHEMOTHERAPY  
AND IMMUNITY TO CYCLICALLY TRANSMITTED  
INFECTIONS IN RABBITS

By

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This thesis is dedicated to  
my parents for their unwavering  
love and encouragement.

DECLARATION

I, Richard Samwel Silayo, hereby declare  
that this thesis was composed by me  
and that the work described,  
was my own.

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## ABSTRACT

This thesis describes studies on curative and prophylactic effects of diminazene aceturate and isometamidium chloride and development of sterile immunity in rabbits infected with Trypanosoma congolense by bites from infected Glossina morsitans morsitans or by intradermal inoculation of cultured metacyclic trypanosomes.

It was shown that chemotherapy influences the development of the trypanosome chancre. Rabbits treated with 7 mg/kg diminazene aceturate at up to three days after infective tsetse bite did not develop chancres, while in animals treated 4-7 days post-infection, chancres appeared but were reduced in size and persisted for a short while. Treatment at 8-10 days after infection did not influence chancre size appreciably.

There were indications that efficacy of chemotherapy was related to the timing of treatment after infection and hence the stage of development of parasites in the trypanosome chancre. Thus 50% of rabbits treated with 7 mg/kg diminazene aceturate at 8-10 days post-infection had relapses in parasitaemia, whereas animals treated with a similar dose of the drug at 0-7 or 12-42 days after infection were invariably cured.

There was indirect evidence that in drug treated rabbits in which relapses in parasitaemia occurred, trypanosomes may have originated from extravascular sites in the skin. This was indicated by development of secondary chancres in some treated animals which later showed relapse parasitaemia.

The mode of action of diminazene aceturate on chancre trypanosomes was studied. There was no detectable increase in parasitaemia, following

treatment at the time of maximum trypanosome development in chancres. This indicated that diminazene aceturate does not act on chancre trypanosomes by causing their release into the circulatory system. In addition, intact and degenerating trypanosomes could be detected histologically in chancres removed at three hours and at 24 hours post-treatment but not at 2-4 days post-treatment. As there was no evidence of phagocytosis of trypanosomes it was concluded that diminazene aceturate kills chancre trypanosomes in situ.

Studies on the prophylactic effects of diminazene aceturate and isometamidium chloride showed that 7 mg/kg diminazene aceturate has no prophylactic effect against cyclically transmitted T. congolense even at three hours after treatment, and isometamidium chloride had a prophylactic effect of less than three weeks. In some animals under isometamidium or diminazene prophylaxis, chancres developed without subsequent development of parasitaemia.

Immunity to homologous challenge with T. congolense after infection and treatment with diminazene aceturate was shown to depend on the timing of treatment in relation to the primary infection. Rabbits treated at day 0-12 days after infective tsetse bite were susceptible, whereas those treated at 14 or more days after infective tsetse bite, when parasitaemia was readily detectable, were immune. Similar results were obtained using cultured metacyclic trypanosomes. It was shown that effective immunity against homologous challenge by infected tsetse flies developed in animals treated at 14 days but not at 7 days after infection with cultured metacyclic trypanosomes. This development of immunity following inoculation of cultured metacyclic trypanosomes points to their potential use in immunisation against trypanosomiasis.

## ABBREVIATIONS USED IN THE TEXT, TABLES AND FIGURES

CF1	A strain of randomly bred albino mice originally from Shell Laboratories
CTVM	Centre for Tropical Veterinary Medicine
DE52	Diethylamino-ethyl cellulose
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
E <sub>492</sub>	Extinction at 492 nm
FBS	Foetal Bovine Serum
HPF	High Power Field
IFAT	Indirect Fluorescent-antibody test
ILRAD	International Laboratory for Research on Animal Diseases
I.U.	International Units
LUMP	London University Medical Protozoology
MED (ED <sub>80</sub> )	Minimum Effective Dose (The dose which clears trypanosomes from 80% of the animals treated)
MCD (CD <sub>80</sub> )	Minimum Curative Dose (The dose which cures 80% of animals treated)
NIT	Neutralization of Infectivity test
NZW	New Zealand White (Rabbit)
OPD	Orthophenylenediamine
PBS	Phosphate Buffered Saline
PBSG	Phosphate Buffered Saline Glucose
PCV	Packed Cell Volume
S-RIA	Solid radioimmunoassay
TREU	Trypanosomiasis Research Edinburgh University
VAT	Variable Antigen Type
g	gram

<u>g</u>	gravity
s.d.	standard deviation
Microhaematocrit/ buffy coat technique	Microhaematocrit, darkground/phase contrast buffy coat technique (Murray <u>et al.</u> , 1977)

## CHAPTER ONE

## INTRODUCTION

Few diseases of man and his domesticated animals have as much influence on health and economic development as the tsetse-borne trypanosomiasis of Africa. About 35 million people and 25 million cattle are exposed to the risk of trypanosomiasis in countries affected by the disease (Anon, 1979). Some seven million square kilometres of land is available within the general area of tsetse distribution between latitudes 15°N and 21°S, on which, potentially, an extra 120 million cattle or 400 million small ruminants could be raised if the risk of the tsetse-transmitted trypanosomiasis could be removed (Finelle, 1974; 1980; Griffin, 1978). Even if only a fraction of this potential increase in livestock production could be realised due to other constraints such as alternative land-use priorities, scarcity of water, malnutrition and other infections, any increase would go a long way to improving the general nutrition in countries where the annual per capita consumption of animal protein is below the level required for normal growth and development (Jasiorowski, 1972).

The tsetse-transmitted trypanosomes are parasites of wild animals which infect man and his domesticated livestock when they intrude into the tsetse wildlife ecosystem. "Tsetse-fly" is the common name for flies of the genus Glossina, which are found only in Africa. There are 22 species of Glossina, all potentially capable of transmitting trypanosomes (Mulligan, 1970; Ford, 1971). Each Glossina sp. occurs in a fairly well-defined vegetational zone and feeds preferentially on a small number of different species of wildlife. Hence, some species

of tsetse fly are more important than others in terms of disease transmission to man and domestic animals. Flies belonging to the morsitans group (G. morsitans, G. swynertoni, G. longipalpis, G. pallidipes and G. austeni) are particularly important in disease transmission because they are widely distributed in savannah areas where contact with wild animals, man and domestic animals is facilitated. Other Glossina spp. important in transmitting trypanosomes belong to the palpalis group (G. palpalis, G. fuscipes and G. tachinoides) which is associated with riverine-type vegetation. The third group of tsetse flies, the fusca group which includes G. fusca of forest-edge areas and G. tabaniformis of riverine-forest areas is the least important in disease transmission because there is generally less opportunity for contact with man and his domesticated animals.

All trypanosomes are classified under the genus Trypanosoma Gruby, 1843. The trypanosomes affecting mammals have been divided into two major groups, namely Section Stercoraria and Section Salivaria, according to their mode of development and route of transmission by the vectors (Hoare, 1972). The tsetse-borne trypanosomes belong to Section Salivaria whose members are transmitted by inoculative means in the saliva in the course of feeding by the vector.

The infections caused by tsetse-borne trypanosomes are generally known as the African trypanosomiases. Each infection by a particular trypanosome species in a particular animal host can be regarded as a distinct disease entity (Mulligan, 1970; Losos and Ikede, 1972).

Man is susceptible to two sub-species of Trypanosoma brucei, sometimes regarded as nosodemes of each other. T. brucei rhodesiense causes an acute form of human trypanosomiasis in East and Central Africa while



T. b. gambiense causes a more chronic form of disease in West Africa.

Cattle and pigs are the most important livestock affected by tsetse-borne trypanosomiasis. The pathogenic species of trypanosomes involved are T. vivax and T. congolense in cattle and T. simiae in pigs. T. brucei spp are generally regarded to be of little pathogenic importance in both cattle and pigs but may be important in the epidemiology of human trypanosomiasis since T. brucei rhodesiense has been isolated from cattle while isoenzyme studies have incriminated the pig as a reservoir host of T. b. gambiense (Onyango et al., 1966; Mehltitz et al., 1982). Dogs, equines and small ruminants are more affected by T. brucei spp.

The animal trypanosomiasis affect human health in that losses in meat and milk production lead to protein deficiencies and associated diseases of malnutrition. Even on purely medical grounds, the effects of animal trypanosomiasis are possibly of a greater problem than human trypanosomiasis (Willett, 1970).

Animal trypanosomiasis can result in deaths and abortions, but more frequently, direct losses are due to chronic disease resulting in infertility, reduced growth rate and milk production. Indirect losses result from limitations in the use of imported breeds and draught animals (Finelle, 1980).

It is generally held that trypanosomiasis has contributed to the preservation of natural resources, particularly in areas where G. morsitans is prevalent and it has been suggested that ill-considered and unplanned control of trypanosomiasis in livestock is a potential threat to grazing lands because of the likely explosive increase in cattle population. The consequent overgrazing may contribute to an increase

in surface albedo which has been linked with a decrease in Sahelian rainfall (Ormerod, 1976). On the other hand, it has been argued that tsetse eradication and trypanosomiasis control will provide additional grazing areas thus reducing the overgrazing problem in the currently tsetse-free areas (MacLennan, 1980).

Trypanosomiasis control measures are aimed either at destruction of the vector or at eliminating trypanosomes in the host, but usually the two measures are carried out simultaneously. In the past, tsetse control schemes have included bush clearing to render the habitat unsuitable and extermination of wild animals known to be preferential hosts of the tsetse to remove a food source. Bush clearing for trypanosomiasis control has declined due to rising costs and fear of adverse environmental effects (Finelle, 1974; MacLennan, 1980). Its present use is in the creation of barriers to fly advances into areas where eradication is being carried out by other means. Extermination of wild animals, even on a selective scale, is no longer acceptable on account of its ineffectiveness, unless carried out rapidly, and because of mounting concern for the preservation of wildlife (Finelle, 1974).

At present, the most commonly used method of tsetse control is the application of insecticides from the ground and/or from the air. Considerable success with this method has been obtained in Nigeria and Botswana (Finelle, 1980). Elsewhere, success has been limited by cost and difficulties in preventing reinvasion of pockets of land from which tsetse have been eradicated. In addition, chemicals applied cause unwanted pollution and may adversely affect non-target species (Finelle, 1974; 1980). New methods for controlling tsetse flies are

under experimentation. The most promising are the sterile insect release method (Dame et al., 1980; Williamson et al., 1983) and the use of pheromones and tsetse traps (Langley, 1983; Anon, 1984).

Chemotherapy and chemoprophylaxis constitute the other commonly used method for controlling trypanosomiasis and there has been an increasing demand for the use of trypanocidal drugs (MacLennan, 1980). Unfortunately effective drugs available for this purpose are few in number and for nearly all of them drug resistance is already a problem.

Since 1960, no new trypanocidal drug has gone beyond the experimental stage (Anon, 1979). The development of new drugs for field use is becoming increasingly more difficult and costly. This is largely due to the lack of metabolic leads for drug design, making the search for new compounds necessarily empirical (Williamson, 1970). Even modes of action of the currently used drugs are not known with certainty.

In the past, the efficacy of the different trypanocides against the different salivarian trypanosome species has been related to the distribution of trypanosomes in the body. In general, trypanocides have been more effective against the haematic parasites T. congolense and T. vivax than against the humoral T. brucei group, possibly because of the lower levels of drug found in tissue fluids than in plasma (Williamson, 1970; Losos and Ikede, 1972). However, the recent finding that T. congolense occurs extravascularly in the early stages of cyclical infection points to the possibility of drug failure due to causes other than drug resistance (Gray and Luckins, 1980; Emery and Moloo, 1981).

The success of chemotherapy and chemoprophylaxis as a method of trypanosomiasis control is also limited by the cost of the drug and its application. Many control schemes involving the use of chemotherapy are hampered by erratic supplies of imported drugs, syringes and needles (Holmes and Scott, 1982). Indeed, the inability of affected countries to purchase trypanocidal drugs has made development of new trypanocidal drugs an unattractive venture for drug companies (Williamson, 1976).

Another problem associated with the use of chemotherapy and chemoprophylaxis is the possible presence of drug residues in meat and milk. This is particularly true of phenanthridium compounds (Anon, 1979). For example, Braide and Eghianruwa (1976) have shown that isometamidium can still be detected in liver and kidney four weeks after treatment designed to protect animals en route to slaughter. The long term effects in humans consuming products with trypanocidal drug residues has not been investigated.

Immunological methods of controlling trypanosomiasis would be preferable to the use of chemotherapy because of its lower cost and lack of residue problems. The methods currently being considered are the use of trypanotolerant animals, vaccination and the use of chemotherapy to foster immunity.

Trypanotolerant breeds of livestock are those which, despite infection with trypanosomes show few clinical signs of disease. They include game animals and the indigenous West African humpless Bos taurus cattle. The breeds of cattle in which trypanotolerance has been observed and studied in most detail are N'dama and Muturu. These breeds are known to develop lower parasitaemias than susceptible breeds of

Bos indicus. However, trypanotolerant animals acquire and maintain their resistance to disease only when subjected to constant challenge from trypanosome-infected flies (Murray et al., 1979; 1982; Vickerman and Barry, 1982). In addition, trypanotolerance may break down in the face of high challenge or other stress factors. Other disadvantages of the utilization of trypanotolerant animals include limitation of breeding stock and possible unacceptability of the breeds by pastoralists more used to the larger Zebu cattle.

Immunization against trypanosomiasis would be the ideal method for controlling the disease, but presently vaccination appears to be a method of the distant future. This is due to the complexity of trypanosome antigens. In both metacyclic and bloodstream stages trypanosomes are covered with a protective glycoprotein surface coat containing variant antigens which alter throughout the course of infection in the vertebrate host (Vickerman, 1969a; 1978). Antibodies are produced against the main antigenic type (homotype) of a population of trypanosomes which is heterogenous antigenically. When the homotype is eliminated by antibody, one of the heterotypes multiplies and becomes the new homotype against which more antibodies are produced. This process, which continues throughout infection, is known as antigenic variation. Animals can develop immunity against trypanosomiasis, but it is specific to the variable antigen types (VATs) inoculated. The number of VATs which can appear in the course of infection with a single trypanosome has been said to be limited only by the duration for which the infected host survives (Gray, 1965b). For example, the number of antigens expressed by a clone of T. equiperdum has been shown to exceed one hundred (Capbern et al., 1977).

The hope that animals may be vaccinated against trypanosomiasis in spite of antigenic variation came from the early findings that when trypanosomes develop in the tsetse fly each serodeme reverts to what has been termed "basic antigens" (Broom and Brown, 1940; Gray, 1965a). In addition, in the early stage of infections transmitted mechanically by syringe, certain antigen types termed "predominant antigens" tended to appear in a predictable manner (Gray, 1965b). Thus, the choice of antigens to be used in vaccination could possibly be restricted to those comprising basic antigens. However, basic antigens are serodeme specific and it is not known how many different serodemes of each trypanosome species exist in nature. In addition, methods of obtaining large amounts of metacyclic trypanosomes are still under experimentation. Tsetse flies extrude only small numbers of metacyclics and thus cultural methods would have to be resorted to, in order to produce the numbers required. There has been success in culturing metacyclics of T. brucei, T. congolense and T. vivax (Hirumi et al., 1977; 1983; Gray et al., 1981) but many more stocks have to be cultivated before antigens for vaccination can be selected.

In the past, experimental immunization of animals has been carried out using various procedures. These have included inoculation of killed or irradiated bloodstream forms as well as infection and treatment (Reviewed by Gray and Luckins, 1976; Murray and Urquhart, 1977; Holmes, 1980). Infection and treatment has been the most interesting technique because of its possible application in the field. Laboratory studies have shown that following chemotherapy, cattle are immune to the serodemes of trypanosomes which initiated the infections (Cunningham, 1968; Wilson, 1971). Field studies have shown that cattle

undergoing treatment acquire immunity, but this depended on the drug regime (Wilson et al., 1975; 1976). Animals treated on an individual basis after showing signs of the disease developed immunity while those treated as a group whenever one animal in the group required treatment, never developed immunity.

Relatively little work has been done on the development of immunity to trypanosomes following drug treatment particularly in animals with cyclically transmitted infections. Syringe transmitted infections of bloodstream forms of T. congolense in cattle have been shown to differ from cyclically-transmitted infections in that following bites by infected flies, local skin reactions or chancres develop, whereas bloodstream forms do not induce chancres (Akol and Murray, 1982). This has also been shown to be the case in rabbits (Luckins, Gray and Rae, 1983). The local skin reaction which develops at the site of infective tsetse bites is a site of localisation and multiplication of the metacyclics extruded by the probing tsetse fly (Willett and Gordon, 1957; Gray and Luckins, 1980; Emery and Moloo, 1981). It is possible that abrogation of the chancre could influence subsequent development of immunity. For instance, in cattle it has been shown that treatment with diminazene aceturate at 15 days post-infection leads to development of immunity while treatment at 5 or 10 days after infection was ineffective (Emery et al., 1980). However, other studies have indicated that in rabbits treated with homidium chloride at day 7 after cyclical infection, long lasting immunity develops (Luckins, personal communication 1981; Luckins, Rae and Gray, 1983).

In the following work, some aspects of the effects of chemotherapy on infections with T. congolense in rabbits were investigated, in

order to provide insight into some of the factors influencing efficacy of treatment and development of immunity in animals infected with cyclically-transmitted stocks of trypanosomes.



## CHAPTER TWO

## LITERATURE REVIEW

2.1 Classification and Nomenclature

Trypanosomes are classified under the genus Trypanosoma Gruby, 1843 and are members of the sub-kingdom Protozoa, order Kinetoplastida Honigberg, 1963 emend Vickerman, 1976, and family Trypanosomatidae Doflein, 1901 emend Grobben, 1905 (Anon, 1980; Molyneux and Ashford, 1983). Table 2.1 (After Molyneux and Ashford, 1983) sets out the systematic classification of the various members of the family Trypanosomatidae.

2.1.1 Genus Trypanosoma Gruby, 1843

The genus Trypanosoma Gruby, 1843 comprises flagellates which exist as digenetic parasites of vertebrates and haematophagous invertebrates. The type species is the frog trypanosome Trypanosoma rotatorium (Mayer, 1843) Gruby, 1843, previously named Amoeba rotatoria and Trypanosoma sanguinis in 1843 by Mayer and Gruby respectively (Hoare, 1972). Hoare (1964) proposed the now widely accepted grouping of different species of trypanosomes of mammals into two sections, Stercoraria and Salivaria according to their mode of development in the vector.

The section Stercoraria contains species in which transmission is by contaminative means, occurring when infected vector faeces are deposited on abraded skin or on mucous membranes of the host. It contains only one species of pathogenic importance, Trypanosoma (Schizotrypanum) cruzi which causes Chagas' disease (also known as American trypanosomiasis) of man in Central and South America. The cosmopolitan species T. (Megatrypanum) theileri of cattle and T. (M) melophagium of sheep, though non-pathogenic may have diagnostic importance.

Trypanosomes in section Salivaria are transmitted by inoculative means in the saliva of the vector in the course of feeding. They are classified into four subgenera according to their morphology in the mammalian host and whether developmental sites in the insect vector include proboscis only (subgenus Duttonella), midgut and proboscis (subgenus Nannomonas) or midgut and salivary glands (subgenera Trypanozoon and Pycnomonas). The type species are T. (Duttonella) vivax, T. (Nannomonas) congolense, T. (Trypanozoon) brucei and T. (Pycnomonas) suis. In the subgenera Duttonella and Trypanozoon, there are species which have lost dependence on cyclical transmission by Glossina and are capable of being transmitted non-cyclically. T. (D) vivax viennei and T. (T) evansi may be transmitted mechanically by biting flies such as Tabanus and Stomoxys while T. (T) equiperdum is transmitted venereally.

(a) Speciation in section Salivaria: The criteria for classifying trypanosomes to the species level are still rather inadequate in spite of recent developments in isoenzyme characterization, particularly with the trypanosomes responsible for causing trypanosomiasis in man in both the Old and New Worlds. Speciation is still assessed on the basis of morphology, host specificity and pathogenicity. Hoare (1972) argues that on the basis of the intergradation of behavioural characteristics each subgenus in the section Salivaria should be regarded as monospecific but for practical purposes the previously recognised and long accepted speciation in the two subgenera Duttonella and Nannomonas are retained; hence T. (D) vivax, T. (D) uniforme, T. (N) congolense and T. (N) simiae. On the other hand, in the subgenus Trypanozoon, Hoare (1972) recognises three species, T. evansi, T. equiperdum and T. brucei with three sub-

species, T. brucei brucei, T. b. rhodesiense and T. b. gambiense which were previously recognised as separate species.

Due to the inadequate present criteria for speciation it has been recommended that each salivarian subgenus be regarded as unispecific with subspeciation being allowed for on the basis of behaviour in hosts of special interest such as man and domesticated animals (Anon, 1979). Accordingly, a Salivarian species has been defined as an assemblage of organisms which can be distinguished from other species by one or more stable discontinuous morphological characters, while a Salivarian subspecies has been defined as an assemblage of organisms within a species, which cannot be separated from each other by morphological characters, but only by other stable characters (Anon, 1979). However, to date, the recommendation that each salivarian subgenus be regarded as unispecific does not appear to have been put into practice. Table 2.2 shows a recent classification of mammalian trypanosomes, still based on the recommendations of Hoare (1972).

(b) Morphological identification of salivarian species: The morphological characters on which speciation of salivarian trypanosomes is based are those of the trypomastigote bloodstream form. These forms are basically lanceolate in shape, resembling an elongated flattened blade which is elliptical or oval in the transverse sections while its ends taper to a point. The diagnostic features used for species differentiation are total length, position and size of kinetoplast, presence or absence of a free flagellum and extent of convolution of the undulating membrane (Hoare, 1972). These features are best seen in Giemsa-stained smears. The morphological features of the different salivarian species are described by Hoare (1970; 1972).

Table 2.1

Classification of Kinetoplastida.

Kingdom	Protista
Subkingdom	Protozoa
Phylum	Apicomplexa Sarcomastigophora Microspora Ciliophora
Subphylum	Opalinata Mastigophora Sarcodina
Class	Phytomastigophorea Zoomastigophorea
Order	Kinetoplastida
Suborder	Bodonina Trypanosomatina
Family	Trypanosomatidae
Genus	<i>Crithidia</i> <i>Leptomonas</i> <i>Herpetomonas</i> <i>Blattocrithidia</i> <i>Trypanosoma</i> <i>Phytomonas</i> <i>Leishmania</i> <i>Endotrypanum</i> <i>Rhynchoidomonas</i>

(After Molyneux and Ashford, 1983)

Table 2.2

Classification of the genus *Trypanosoma*.

Section		<i>Trypanosoma</i>			
		Stercoraria		Salivaria	
Subgenus		<i>Megatrypanum</i>	<i>Herpetosoma</i>	<i>Schizotrypanum</i>	<i>Duttonella</i>
Representative species		<i>T. (M.) theileri</i>	<i>T. (H.) lewisi</i> <i>T. (H.) musculi</i> <i>T. (H.) rangeli</i>	<i>T. (S.) cruzi</i>	<i>T. (D.) uniforme</i> <i>T. (D.) vivax</i>
				<i>T. (N.) congolense</i> <i>T. (N.) simiae</i>	<i>T. (T.) brucei</i> <i>T. (T.) b. brucei</i> <i>T. (T.) b. rhodesiense</i> <i>T. (T.) b. gambiense</i> <i>T. (T.) equiperdum</i> <i>T. (T.) evansi</i>
					<i>Pycnomonas</i>

(After Molyneux and Ashford, 1983)

In the insect vector salivarian trypanosomes exist as trypomastigote and epimastigote forms. In the vertebrate host only the trypomastigote stage exists but there are claims that other morphological types such as amastigotes can occur.

#### 2.1.2 Subgenus Nannomonas Hoare, 1964

Trypanosomes of the subgenus Nannomonas are represented by relatively small organisms measuring 8-24  $\mu\text{m}$  in total length and are characterized by a subterminal, marginal kinetoplast, an inconspicuous undulating membrane and lack of a free flagellum (Hoare, 1972).

(a) Trypanosoma congolense Broden, 1904: In 1904, Broden working in Leopoldville, Congo, found unusually small trypanosomes without a free flagellum, in the blood of sheep and a donkey. The name T. congolense was given to this new trypanosome species (Hoare, 1972). The historical aspects of its nomenclature (Reviewed by Hoare, 1972) is interesting particularly with regard to the changing status of T. dimorphon now regarded as a synonym of T. congolense.

T. dimorphon Laveran and Mesnil, 1904, was the name given to a dimorphic trypanosome isolated in a rat which had been inoculated with blood of a horse from the Gambia (Hoare, 1972). It was differentiated from T. congolense on the basis of its greater mean length and the proportions of long, short and intermediate forms.

Hoare (1970) suggests that because of the inadequate first description of T. dimorphon, the name was frequently misused and trypanosomes of both types were for many years cited indiscriminately as T. congolense and/or T. dimorphon. T. dimorphon gradually passed out of general usage and became a synonym of T. congolense. In a morphological

and biometric revision of T. dimorphon and T. congolense based on the original material studied by Laveran and Mesnil in 1904, Hoare (1959) restored T. dimorphon to the status of a distinct species. This decision was based on the finding that statistical analysis of the mean lengths showed a definite difference between the mean lengths of T. congolense (12.2-14.4  $\mu\text{m}$ ) and T. dimorphon (15.3-17.6  $\mu\text{m}$ ).

Godfrey (1960) and Fairbairn (1962) found stocks of T. congolense with mean lengths between those of T. congolense and T. dimorphon. It was proposed that T. congolense be subdivided into three distinct subgroups, the congolense type, the intermediate type and the dimorphon type based on the differences in mean lengths (Godfrey, 1960; Fairbairn, 1962).

Huisenga (1969), in another study of the original material of Laveran and Mesnil (1904) and material supplied by Hoare, found that when measured exclusively in the initial stages of division, both T. congolense and T. dimorphon have the same mean lengths, implying that there is no justification for distinguishing subspecies or types on the basis of mean lengths. Hoare (1972) interpreted this finding as showing that in all stocks of T. congolense divisions are restricted to individuals of the same mean lengths and that these trypanosomes then differentiate into adult trypanosomal populations conforming to congolense, intermediate or dimorphon types.

Nantulya, Doyle and Jenni (1978a) showed that the three different morphological forms of T. congolense described by Godfrey (1960) and Fairbairn (1962) could be found at different stages of infections initiated with a single organism. The morphological appearance of the cloned trypanosomes in mice was found to undergo gradual change in the

course of a parasitaemic wave. On day 5 after infection, the picture was that attributable to intermediate type. At peak parasitaemia seven to eight days after infection the trypanosome population was highly pleomorphic with long forms predominating and the overall picture was that attributable to a dimorphon type of T. congolense. Rapid syringe passage at 3-4 day intervals resulted in the trypanosome population transforming to a short, congolense type.

(b) Trypanosoma simiae Bruce et al., 1912: T. simiae was the name given by Bruce et al. (1912) to a trypanosome isolated from a monkey infected by bites from wild caught tsetse flies. A short time later, a trypanosome now known to be the same species was similarly isolated by Kinghorn and York (1912) and named T. ignotum. After it was demonstrated that T. simiae was very virulent to domestic pigs, reports of its natural occurrence started appearing. Stephen (1966) and Hoare (1972) have reviewed the nomenclature of this trypanosome. From the literature, it appears that T. simiae has been distinguished from T. congolense by its greater mean length (15.3-19.7  $\mu$ m) compared to that of T. congolense (11.2-15.7  $\mu$ m), the marked pathogenicity of T. simiae for the pig and its lack of infectivity for cattle, mice and rats. Also, T. simiae is regarded as refractory to most chemotherapeutic drugs available.

There are now doubts as to the validity of T. simiae as a distinct species on present criteria. Roberts (1971) found that the length of T. simiae was influenced by the host species. A behaviourally defined T. simiae when passaged in sheep was found to have a mean length which fell within the T. congolense range.



Janssen and Wijers (1974) found that the species of Glossina responsible for transmitting T. simiae can also influence the degree of pathogenicity in pigs. Thus when a stock of T. simiae was transmitted by G. brevipalpis it was more virulent to pigs than the same stock when transmitted by G. pallidipes.

(c) Taxonomic status of Trypanosoma congolense and Trypanosoma simiae: Godfrey (1977) on the basis of the morphological and behavioural criteria used to distinguish T. simiae from T. congolense concluded that the subgenus Nannomonas is best regarded as comprising many types which could be divided into organisms that have a strong tendency to be naturally restricted to pigs and those organisms that do not. The morphological and behavioural differences between T. congolense and T. simiae seem to intergrade to such an extent that they can be regarded as clines of a single species (Trypanosoma congolense Broden, 1904) not warranting even subspecific distinction (Anon, 1978). It is for this reason that the subgenus Nannomonas should be regarded as unspecific, with two subspecies T. congolense congolense and T. congolense simiae (Anon, 1979). However, this view has not found popular approval and in the following account the generally accepted nomenclature will be used.

## 2.2 Development of Trypanosoma congolense in the Vector

In nature, the transmission of T. congolense is by bites of infected Glossina spp. Accounts of the complete cycle of development of T. congolense in tsetse flies first appeared as early as 1912 (Reviewed in Hoare, 1972). An outline of development of T. congolense

in Glossina spp. has been given by Hoare (1972). Ingested trypanosomes transform into elongated trypomastigotes and multiply within the endoperitrophic space of the midgut, then escape into the ectoperitrophic space and migrate via the soft proventriculus and oesophagus into the proboscis. Here they attach to the walls of the labium and transform into epimastigotes. These enter the hypopharynx and complete the cycle by transforming into metatrypanosomes, which are infective to the vertebrate host. The whole life cycle was held to take 19-53 days. More recently Nantulya et al. (1978) found that the entire life cycle can take 7-40 days, 45% of infected flies becoming infective by day 12 and 76% by day 18.

Several Glossina spp. are capable of transmitting T. congolense (Hoare, 1972). Infection rates in the field average 2% compared to 5-15% in the laboratory (ILRAD, 1982). The species of Glossina has been shown to influence infection rates. Harley and Wilson (1968) compared G. morsitans, G. pallidipes and G. fuscipes as vectors of T. congolense and found infection rates of 11.6%, 13.2% and 2.9% respectively.

There have been several investigations into factors which influence trypanosome infection rates in tsetse flies. Godfrey (1958, 1959) found that when the temperature at which Glossina pupae were incubated was raised from normal to 28°C the infection rates in the flies rose from 15.5% to 35%. Dipeolu (1975) raised the maintenance temperature for adult G. m. morsitans from 26°C to 31°C and found that this did not influence their susceptibility to T. congolense.

The influence of level of parasitaemia in the infective blood meal on subsequent fly infection rates was investigated and found not

to affect the infection rates (Dipeolu, 1975). Recently, Nantulya et al. (1978b) showed that teneral G. morsitans fed on mice with peak parasitaemia of T. congolense had higher infection rates compared with those fed before or after peak parasitaemia. Infection rates of 7.1%, 22.4% and 6.6% were obtained for flies fed on log phase of rising parasitaemia, peak parasitaemia and following phase of parasitaemic wave respectively.

The influence of the host species on infection rates has also been investigated (Moloo, 1981). G. m. morsitans maintained on goat or rabbit were found to have higher infection rates than those maintained on the rat or mouse.

There is new evidence to show that tsetse flies may be bred for high or low trypanosome infection rates. The maternal phenotype of G. m. morsitans has been shown to influence their susceptibility to T. congolense (Maudlin, 1982).

### 2.3 Development of Trypanosoma congolense in the Vertebrate Host

The initial development of T. congolense in the vertebrate host depends on whether the infective metacyclic forms are deposited intradermally or elsewhere. Cyclically transmitted T. congolense has now been shown to induce local skin reactions, or chancres, at sites where infected tsetse flies have fed (Roberts, Gray and Gray, 1969; Luckins and Gray, 1978; Gray and Luckins, 1980; Emery and Moloo, 1981; Akol and Murray, 1982). Chancres in which trypanosomes undergo multiplication were first seen in T. brucei infections and much of the earlier work on the nature of this local skin reaction was carried out with T. brucei subspecies organisms.

### 2.3.1 Development of trypanosomes in the local skin reaction - the trypanosome chancre

The first description of chancres is usually associated with Graf (Apted, 1970) who described them in man. The lesion appears two to three days after an infective tsetse bite as an erythematous and tender swelling. Later, it becomes a circumscribed rubbery induration which disappears in 2-3 weeks (Anon, 1979). It is now well recognised as the first symptom of sleeping sickness (Gelfand, 1966; Willett, 1966; Apted, 1970).

(a) Chancres caused by *Trypanosoma brucei* subspecies: Early experimental studies in man showed that bloodstream forms of *T. rhodesiense* inoculated intradermally could also initiate trypanosome chancres (Corson, 1932; Fairbairn, 1933). Willett (1956) inoculated human volunteers subcutaneously with *T. brucei* spp. and found that there was a constant mean interval of 2.6 days between appearance of the reaction at the site of inoculation and the first appearance of trypanosomes in the blood.

In studies aimed at determining where metacyclics are deposited by the feeding fly, Gordon, Crewe and Willett (1956) found that saliva was ejected frequently in the course of probing and feeding and was widely distributed in the host tissues throughout a hemispherical area reached by the labium of the feeding fly. Some saliva could be expected to find its way direct into the lumen of the torn capillary but most would remain at the bite site.

Earlier speculation that it is necessary for trypanosomes to develop in the local skin reaction before they can invade the bloodstream was disproved by the studies of Willett and Gordon (1957). Rats

inoculated with T. brucei intradermally or subcutaneously and guinea pigs bitten by infected tsetse flies did not develop chancres, but the general circulation was rapidly invaded. Intravenous inoculation of metacyclics of T. rhodesiense into rabbits lead to persistence and multiplication of trypanosomes in the blood. Rabbits, unlike rats and guinea pigs, were shown to develop typical chancres when bloodstream forms of T. rhodesiense were inoculated intradermally: the greater the number of trypanosomes inoculated, the earlier the chancre tended to appear. From these studies it appears that there is no evidence of any process or cycle of development from metacyclic to bloodstream form, other than the direct conversion of the former to the latter and their subsequent multiplication by binary fission (Willett and Gordon, 1957).

Fairbairn and Godfrey (1957) carried out a single experiment in which T. rhodesiense-infected tsetse flies were allowed to feed on a volunteer whose blood was subinoculated into rats one hour later, and daily thereafter up to day 10. Positive subinoculations were obtained starting from day 9 post-infection when a typical chancre was seen at the site of tsetse bite. Puncture fluid from this reaction revealed bloodstream forms of T. rhodesiense; a biopsy taken for histological examination on day 10 revealed trypanosomes and cellular reaction in the dermis. All these findings were considered as evidence that in man, metacyclics inoculated by the tsetse fly remain at the site of inoculation, multiply and develop into blood forms which later overflow into the general circulation.

Vickerman (1969a) suggested that the surface coat which metacyclic trypanosomes acquire while in the fly, preadapt them for life in the

bloodstream without needing to adapt extravascularly in the trypanosome chancre. Barry and Hajduk (1979) injected single metacyclics of T. brucei intravenously into rabbits and found that infections developed in all five rabbits inoculated, thus corroborating Vickerman's hypothesis.

Early studies showed that trypanosomes multiply in the chancre. Willett and Gordon (1957) observed that in rabbits bitten by an infected tsetse fly, trypanosomes were not seen at the site of inoculation until 72 hours after the bite. The trypanosome counts, in tissue from chancres which subsequently developed, increased both in number and density to concentrations exceeding  $1 \times 10^8$ /ml in histological sections of skin obtained at day 5-7 after the infective tsetse bite.

(b) Development of Trypanosoma congolense in local skin reactions:

The development of local skin reactions to infections with T. congolense in domestic animals was first reported by Roberts et al. (1969), in cattle and sheep, at sites bitten by experimentally infected G. morsitans and G. tachinoides as well as wild-caught G. morsitans. Reactions appeared 4-12 days before patent parasitaemia as determined by microscopical examination of wet blood smears. Chancre fluid examined microscopically revealed trypanosomes morphologically indistinguishable from bloodstream forms and also organisms similar to forms from the midgut and proboscis of the infected tsetse fly.

T. congolense is usually regarded as a strict haematic parasite (Losos and Ikede, 1972; Losos, Paris, Wilson and Dar, 1973). The local skin reaction to T. congolense was shown to be an exception to this haematic distribution when it was found by light and electron microscopy

that T. congolense develops in the connective tissue in local skin reactions (Luckins and Gray, 1978). Evidence of trypanosomal multiplication in this site was provided by the presence of dividing trypanosomes.

Gray and Luckins (1980) carried out extensive histological studies of chancres induced by T. congolense in rabbits, calves and sheep. Chancres removed from rabbits at day 7 post-infection revealed numerous trypanosomes associated with, or inside collagen bundles in the deep dermis. More trypanosomes were seen in reactions removed at day 9 post-infection but by day 11 the number was decreasing. A similar trend was seen in reactions from calves at 8, 10 and 12 days after infection.

Emery and Moloo (1981) quantified both the development of trypanosomes and the cellular reaction in local skin reactions from goats infected with T. congolense. Between days 7 and 15 after infection moderate numbers of parasites were seen between collagen fibres and throughout the inflammatory oedema. In histological studies of T. congolense chancres in cattle, trypanosomes were also seen in collagen bundles and inflammatory oedema separating the collagen bundles as well as in dilated lymphatics of pillary dermis and hypodermis (Akol and Murray, 1982). Bloodstream forms of T. congolense, unlike those of T. brucei, failed to induce local skin reactions when inoculated intradermally into the skin of susceptible hosts (Awad, Magaji and Mshelbwala, 1969; Luckins, Rae and Gray, 1981). This has been confirmed in goats (Emery et al., 1980) and in cattle (Akol and Murray, 1982).

Losos and Ikede (1972) and Losos (1979) suggested that trypanosome chancres in domestic animals are an experimental phenomenon since there have been no reports of its natural occurrence in the field. Nevertheless Roberts et al. (1969) induced chancres in experimental cattle using wild-caught G. morsitans infected with T. congolense. Among the factors which could contribute to the lack of field observations of trypanosome chancres in domestic animals, is the interference phenomenon observed by Morrison et al. (1982b), Luckins and Gray (1983) and Luckins, Rae and Gray (1983). It was found that concurrent infection with T. congolense inhibited induction of chancres following bites by flies infected with an antigenically dissimilar stock. Also, the frequent use of chemotherapy in the field may also influence the appearance of trypanosome chancres. However, no experimental observations on this latter aspect have been carried out.

#### 2.3.2 Tissue forms of Trypanosoma congolense

It is not known what causes trypanosomes in the collagen to leave their site of development but Akol and Murray (1982) have suggested that the disappearance of T. congolense from the lesion is related to biological changes associated with their maturation to bloodstream forms.

The route of migration of trypanosomes from the chancre into the bloodstream is probably via the local lymphatic drainage system. Trypanosomes have been observed in the sinuses of lymph nodes of a calf and sheep ten days after cyclical infection when they were absent in blood (Luckins and Gray, 1979b). In contrast, trypanosomes have



been reported to be scarce or absent in the lymph fluid of animals with T. congolense infections initiated by bloodstream forms (Ssenyonga and Adams, 1975; Tizard, Hay and Wilkie, 1978).

Once trypanosomes reach the bloodstream multiplication is continuous and is by binary fission of trypomastigotes (Hoare, 1972). However, there has always been argument for the existence of a secondary stage of development of salivarian trypanosomes involving an amastigote stage in the vertebrate host.

Observation of amastigote, promastigote, epimastigote and multinucleate forms of T. congolense and T. brucei in impression smears from mice and rabbits has been reported (Soltys and Woo, 1969). Forms which passed through filters of pore size 0.8-1.2  $\mu\text{m}$  were shown to be present in liver and spleen but not blood (Soltys, Woo and Gillick, 1969; Soltys and Woo, 1970). These observations have, however, not generally been accepted as evidence of the existence of trypanosome tissue forms (Ormerod, 1979).

Histological evidence for the existence of an amastigote stage in salivarian trypanosomes has been presented (Ormerod and Venkatesan, 1971). These forms were seen in choroid plexuses of T. brucei-infected rats. Recent ultrastructural studies of choroid plexuses of rats infected with T. brucei rhodesiense showed that multiple division of trypomastigotes takes place in the ependymal cells forming the blood-brain barrier (Abolarin et al., 1982). However, the viability and importance of these forms in the trypanosome life cycle has yet to be shown.

Suggestions that an amastigote or a multinucleate stage in the brain may be responsible for relapses after chemotherapy have come

from studies in mice infected with T. brucei and treated with diminazene aceturate alone or in combination with nitroimidazole (Jennings et al., 1977; 1979; 1980). However it has been suggested that these amastigote and multinucleate forms claimed to be the source of relapsing trypanosomes after chemotherapy may actually have little role in the trypanosome life cycle and may simply be the product of degenerate or aberrant morphogenesis (Vickerman and Tetley, 1979). This is also the view of Fiennes (1970) who considers that there is no evidence that any form of tissue development takes place in the vertebrate body analogous to that which occurs with T. cruzi. The forms that many workers have described as amastigote are thus probably degenerating trypanosomes, described as "trypanosomes en boule" by Laveran (1911).

### 2.3.3 Systemic distribution of Trypanosoma congolense

There is evidence that the distribution of T. congolense in the circulatory system is not uniform. Hornby and Bailey (1931) found that there were more trypanosomes in the capillaries than in arteries and veins and that superficial vessels contained more trypanosomes when the animal was cool. A rise in external temperature or treatment with trypanocidal drugs increased temporarily the numbers of trypanosomes in the veins.

Krampitz (1970) reported that in zebu cattle experimentally infected with T. congolense, parasitaemia in the superficial veins could be ten times that in the jugular vein. This concentration of T. congolense in capillaries was confirmed by Banks (1978) who reported that in stained preparations of mesenteric microvasculature of infected rats and rabbits, there were 4-1400 times more trypanosomes than in

cardiac blood. Vessels examined microscopically in the living animal (Banks, 1978) revealed trypanosomes adhering by their anterior ends to the vessel wall. Trypanocidal drugs effective against T. congolense have been reported to release trypanosomes from the sites of adherence on vessel walls, into central blood circulation (Hornby and Bailey, 1931; Maxie and Losos, 1977; Mills, Valli and Forsberg, 1980; Kalu, 1983; Kalu, Ayandele and Aina, 1983).

T. congolense has also been shown to have a predilection for the capillaries of certain organs. Losos et al. (1973) found that T. congolense accumulated in the microvasculature of the brain, heart and skeletal muscles. Mills et al. (1980) reported that cerebral-cortical capillaries in calves infected with T. congolense showed more trypanosomes than capillaries in muscles or liver.

#### 2.4 Pathogenesis of Trypanosoma congolense

African trypanosomiasis in laboratory and domestic animals is characterised by development of anaemia, tissue lesions and immunosuppression. Anaemia has been considered the cardinal sign of African trypanosomiasis (Murray, 1979). Others have suggested that immunosuppression is the single most important pathogenic mechanism in trypanosomiasis, because infected individuals generally die, not as a direct result of trypanosome activity, but as a result of secondary bacterial and viral infection (Tizard, Mellors and Nielsen, 1980).

##### 2.4.1 Anaemia

The anaemia in bovine trypanosomiasis can be divided into three phases (Reviewed by Losos and Chouinard, 1979; Urquhart, 1980). The

initial or acute phase is characterised by rapid fall in packed cell volume due to erythrocyte destruction, possibly accentuated by haemo-dilution from expanded plasma volume (Holmes, 1976). The extent of erythrocyte destruction correlates with parasitaemia and is reversed by chemotherapy. The cause of this erythrocyte destruction is not known precisely. Causative factors thought to be involved are production of a haemolysin, immune complexes, expansion of mononuclear phagocytic system and fever (Losos and Chouinard, 1979; Urquhart, 1980).

The second phase or chronic phase is characterised by arrest of the fall in packed cell volume, through a compensatory response in the production of more cells from the bone marrow. The extent of erythropoiesis does not, however, match the degree of anaemia and the packed cell volume remains low. It is thought that this is due to failure of iron transport from the massive haemosiderin deposits in mononuclear phagocytic system to the erythropoietic centres (Dargie et al., 1979). In contrast to anaemia in the first phase this anaemia is associated with scanty parasitaemia and is not reversed by chemotherapy (Losos and Chouinard, 1979; Urquhart, 1980).

In the third phase of anaemia associated with bovine trypanosomiasis the packed cell volume may return to normal but it can remain low, leading to eventual death of the animal. The low packed cell volume, as in the second phase, is also thought to be due to a failure to re-utilize iron (Dargie et al., 1979).

#### 2.4.2 Immunosuppression

Immunosuppression is now an established feature of African trypanosomiasis. Goodwin, Green, Guy and Voller (1972) showed that

injection of sheep red blood cells (SRBCs) into mice and rabbits infected with T. brucei led to the production of lower anti-SRBC agglutinin levels than in uninfected control animals similarly treated with SRBCs. Numerous studies have confirmed the recurrence of marked depression in antibody responses to heterologous non-trypanosomal antigen in murine trypanosomiasis (e.g. Hudson and Terry, 1979; Roelants et al., 1979; Baltz et al., 1981).

Evidence that immunosuppression also occurs in bovine trypanosomiasis was provided when Holmes et al. (1974) showed that cattle infected with T. congolense developed lower secondary responses to polyvalent clostridial vaccines than uninfected controls. Subsequent studies confirmed the immunodepressive effects of trypanosomal infection in cattle immunized with other antigens including Brucella abortus (Rurangirwa et al., 1979b; 1983), Leptospira biflexa (Rurangirwa, 1979b; 1980b), Mycoplasma mycoides (Rurangirwa et al., 1978; Ilemobade et al., 1982) and Foot-and-Mouth Disease virus (Scott et al., 1977; Sharpe et al., 1982).

The earlier fears that immunosuppression associated with bovine trypanosomiasis may interfere with responses to vaccination have not been confirmed. It has been shown that the primary and secondary antibody responses to foot-and-mouth disease virus vaccine in cattle with naturally acquired trypanosomiasis or experimental T. congolense infections, were generally above the level considered adequate to confer 95% protection against challenge (Scott et al., 1977; Sharpe et al., 1982). With rinderpest vaccination it was shown that infection with T. congolense and T. vivax did not significantly depress the neutralizing antibody response of cattle to live rinderpest virus when

vaccination was carried out at eight or 25 days after infection (Rurangirwa et al., 1980a).

However, immunosuppression may be important in relation to pathogenicity since animals often die as a result of secondary bacterial and viral infection. It may also explain why animals do not rapidly acquire solid immunity to all the local strains of trypanosomes, since it has been shown that trypanosome-infected cattle are also immunosuppressed to concurrent infection with other trypanosome stocks (Nantulya et al., 1982). Similar impairment of antitrypanosomal immune responses have been observed in trypanosomiasis infection in mice (e.g. Sacks et al., 1980; Sacks and Askonas, 1980) and rabbits (Luckins and Gray, 1983; Luckins et al., 1983).

The mechanism of immunosuppression is not known with certainty (Losos and Chouinard, 1979). It is likely that in cattle it requires the presence of live trypanosomes as it has been shown that immune competence is spontaneously restored upon trypanocidal treatment (Rurangirwa, Tabel and Losos, 1979a). In contrast, in mice it has been shown that even killed trypanosomes or their subcellular membrane fractions can cause immunosuppression (Clayton, Sacks, Ogilvie and Askonas, 1979). Other studies have suggested that antigenic competition may be responsible for low antibody response to some clones when cattle are simultaneously or subsequently challenged with antigens of more than one clone of T. congolense or T. brucei (Nantulya et al., 1982).

It has also been suggested that complement depletion in the course of trypanosome infection contributes to the immunosuppression associated with trypanosomiasis. It is now held however, that low complement levels probably contribute more to predisposition of

infected animals to secondary infection rather than actual immunosuppression (Rurangirwa et al., 1979a).

## CHAPTER THREE

## MATERIALS AND METHODS

3.1 Animals

Male and female New Zealand white (NZW) rabbits or Sandylop rabbits obtained from the Centre for Laboratory Animals, Edinburgh University, were used in studies on the development of local skin reactions, immunity, chemotherapy and for maintenance of tsetse flies. Rabbits were used at 8-10 months of age when they weighed 2-3 kg. Each was kept in an individual metal cage with food (Rabbit pellets - S.G.1, Oxoid Laboratory Animal Diets) and water ad libitum. Rabbits were identified by numbered ear tags or by marking with picric acid in the case of animals whose ears were used for maintenance of tsetse flies.

Female, albino, random-bred CF1 mice, obtained from the Centre for Laboratory Animals, Edinburgh University, were used for drug sensitivity assays, for passaging stocks of T. congolense, for infecting tsetse flies and for antigen and stabilate preparation. Mice 6-8 weeks old, weighing 20-30 g were kept in plastic cages with metal tops through which food (Rat cake - Oxoid Laboratory Animal Diets) and water were offered ad libitum. Up to five were kept in one cage on wood shavings and water absorbent granules - "Oakite" (Oakite Ltd., Retford, Nottinghamshire, England). Each animal was individually marked with picric acid.

3.2 Handling of Animals

Intravenous and intramuscular injections into rabbits were carried out with the help of an assistant in restraining the animal. Intramuscular injections in the rear legs were done using a 25 gauge



16 mm needle (Becton Dickinson) into and along the length of the semitendinosus or semimembranosus muscle. For intravenous inoculation and for bleeding from the marginal ear vein, 20 gauge, 25 mm needles were used. For bleeding from the ear vein, rabbits were wrapped up firmly in a towel; blood was collected by allowing it to drop directly into a glass universal bottle or a glass vial containing EDTA. To facilitate bleeding, the ear was rubbed with a little xylene at the distal end to cause distension of the marginal blood vessels.

For routine tail-bleeding and subcutaneous or intraperitoneal injection, mice were handled without anaesthesia. To reduce the chances of accidental intraviscus inoculation in the course of intraperitoneal injection with trypanocidal drugs, mice were starved for 24 hours before use. For collection of blood for subinoculation or stabilation, mice were exsanguinated under ether anaesthesia.

### 3.3 Serum Preparation

Blood for serum preparation was allowed to clot by standing for one hour at room temperature and one hour at 37°C followed by 18-24 hours at 4°C. The serum was then separated from the clot by centrifugation at 1500 g for 30 minutes at 4°C and stored at -20°C until needed.

### 3.4 Trypanosome Stocks

The trypanosome stocks were derived by mouse passage from two stabilates, T. congolense TREU 1457 and T. congolense TREU 1468 which were cloned derivatives of stocks ZARIA/67/LUMP/79 and SERENGETI/66/

LUMP/12 respectively. Stabilates T. congolense TREU 1613, TREU 1690 and TREU 1725 were derived from T. congolense TREU 1457 (Figure 3.1). Stabilate T. congolense TREU 1682 was derived from T. congolense TREU 1468 as shown in the pedigree chart Figure 3.2. Plate 3.1A shows trypanosomes in a Giemsa stained smear from a mouse infected with T. congolense TREU 1457.

### 3.5 Culture Metacyclics

These were derived from T. congolense TREU 1457 and maintained in culture by the methods described by Gray et al. (1981). The culture metacyclics were obtained by courtesy of Dr. C.A. Ross of the Protozoology Section, C.T.V.M. Metacyclic forms were separated from epimastigotes on equilibrated ion-exchange resin Diethylaminoethyl cellulose (DE52, Whatman Chromedia), by elution with phosphate buffered saline glucose pH 8.0 (PBSG) using techniques described by Lanham and Godfrey (1970). Trypanosomes were concentrated by centrifugation at 500 g for 15 minutes at 4°C. Plate 3.1B shows Giemsa-stained culture metacyclics of T. congolense TREU 1457.

### 3.6 Stabilation and Retrieval of Stabilates from Cryobank

Stabilates were maintained in liquid nitrogen. The process of stabilation and retrieval was essentially that described by Lumsden, Herbert and McNeillage (1973). Mice used to subpassage trypanosomes for stabilation were immunosuppressed by treatment with 300 mg/kg cyclophosphamide (Koch-Light) intraperitoneally, 18-24 hours before infection. Trypanosomes were subpassaged at 3-6 day intervals and stabilized when mice had developed a fulminating parasitaemia.

Blood for stabilation was collected from the hearts of mice under ether anaesthesia into a 1.0 ml syringe containing 0.1 ml heparin (50 IU/ml). Usually 0.5-1.0 ml of blood could be obtained from one mouse. The volume of blood was noted and then transferred into sterile bijoux bottles kept cool in an ice-bath. Sufficient 50% glycerol in phosphate buffered saline (PBS) pH 8.0 was then added to the cooled blood with a micropipette (Gilson Pipetman, Anachem Ltd., Luton, England), to achieve a glycerol concentration of 7.5%. After gentle mixing the blood was transferred into sterile glass capillary tubes 100 mm long, using a Pasteur pipette. The capillary tubes were held in a metal rack kept on an ice-cold slab (KRYRACK - Streck Laboratories Inc., Omaha, NE, U.S.A.), during the filling and sealing processes. Tubes were filled with blood up to two-fifths of the total length and were then transferred into a perforated plastic tube which was immediately transferred into an insulating jacket (Lumsden et al., 1973) for slow cooling overnight in an ice-box containing solid carbon dioxide.

After 18 hours at  $-79^{\circ}\text{C}$  the plastic tube containing capillary tubes was transferred onto a pre-cooled metal cane which was immediately placed into a canister in a liquid nitrogen flask. A sample capillary tube was removed and checked for motility and viability of the trypanosomes. Stabilates were given TREU numbers and documented as suggested by Lumsden et al. (1973).

For initiating infections in mice, the contents of a capillary tube were diluted with phosphate buffered saline containing 1% glucose (PBSG), pH 8.0.

### 3.7 Monitoring Parasitaemia

Parasitaemias in mice were monitored by examination of tail blood by phase contrast microscopy using a x40 objective, x10 ocular in a Vickers M15C Microscope (Vickers Instruments, England). The quantity of blood used was just sufficient to fill the area of a 6 x 22 mm coverslip. At least 20 high power fields (HPF) were examined with the x40 objective for the presence of trypanosomes.

In rabbits, parasitaemia was monitored by the microhaematocrit phase-contrast, buffy coat technique (Murray, Murray and McIntyre, 1977). Peripheral blood was collected from the ear vein into heparinised glass capillary tubes (Heparinised Benjamin Haematocrit Tubes, Harshaw Chemicals Ltd., Daventry, England) which were sealed at one end with plasticine and then centrifuged for four minutes in a microhaematocrit centrifuge (Hawksley, England). A section of the capillary tube containing the buffy coat together with red blood cells up to 1 mm below and plasma up to 30 mm above was cut with a diamond tipped pencil and the contents expressed onto three areas on a slide, mixed and covered with 22 x 22 mm coverslips. At least 20 HPFs were examined in the area of each coverslip.

### 3.8 Separation of Trypanosomes from Blood and Cultures

One hundred grams of pre-swollen (DE52) was equilibrated by mixing with 5 litres PBS pH 8.0 and adjusting the pH of the resulting suspension with 5% v/v Analar orthophosphoric acid (British Drug Houses). When equilibrated to pH 8.0 the ion-exchange cellulose suspension was stored at +4°C for short periods or at -20°C for longer periods.

When required for separation, equilibrated DE52 was packed with Whatman 3-piece Filtration Unit in which a Whatman No. 4 filter paper had been placed. Packing was done by allowing fluid from the cellulose suspension to drain away slowly, aiming at obtaining a horizontal top surface. This surface was then covered with another Whatman filter paper No. 4. PBSG was then run through and the pH of the eluate checked to confirm that it was registering pH 8.0 before applying blood or culture medium containing trypanosomes.

Blood collected from mice with fulminating infections was immediately diluted 50:50 v/v with ice-cold PBSG and then layered on top of the equilibrated DE52. Trypanosomes were eluted with ice-cold PBSG collected in a sterile polystyrene universal bottle, and then concentrated by centrifugation at 4°C for 30 minutes at 1500 g.

Cultured metacyclic trypanosomes were separated by applying culture fluid containing trypanosomes onto the top of equilibrated DE52 column and eluting with PBSG as above. The separated culture metacyclics were then concentrated by centrifugation at 1500 g for 15 minutes.

### 3.9 Trypanocidal Drugs

Three drugs were used in this work: Diminazene aceturate B. vet.C. (BERENIL, Farbwerke Hoechst AG, Frankfurt-Main), Homidium bromide B.vet.C. (ETHIDIUM, The Boots Company Ltd., Nottingham, England) and Isometamidium chloride B.vet.C. (SAMORIN, May and Baker Ltd., Dagenham, England).

Diminazene aceturate was a gift from Hoechst U.K. Ltd. (courtesy of Mr. J.M.B. Best, Marketing Manager). It was supplied in 2.36 g

sachets containing 1.05 g active principle, the rest being an anti-pyrene used as stabilizer to extend the shelf life of made-up solutions. However, in all experiments only freshly prepared solutions were used. For rabbits, solutions containing 3.5% active principle were prepared in 5 or 10 ml volumetric flasks. For mice, 0.01-0.20% solutions were prepared in 10, 20 or 25 ml volumetric flasks and diluted so that the required dose per gram body weight was contained in 0.01 ml.

In the rabbit, drugs were administered by deep intramuscular injection, dividing the dose so that each hindlimb received less than 0.6 ml. In the mouse the drug was inoculated intraperitoneally.

Homidium bromide was supplied as tablets containing 250 mg of active principle. Each tablet was crushed and then dissolved in distilled water at room temperature, for preparation of 2.5% solutions in volumetric flasks. Fresh drug solutions were used in all experiments. Route of administration in the rabbit was by deep intramuscular injection, the dose being divided so that each hindlimb received less than 0.6 ml. Mice were inoculated intraperitoneally.

Isometamidium chloride was a gift from May and Baker Ltd., Dagenham, England (courtesy of Mr. D.A. Hutchings). It was used as a 1% solution made up by dissolving the drug in water at room temperature in 5 or 10 ml volumetric flasks. It was used only in rabbits and was administered by deep intramuscular injection as described above.

### 3.10 Tsetse Flies

G. m. morsitans pupae were supplied by courtesy of Dr. A.M. Jordan, Tsetse Research Laboratory, Langford, Bristol. On receipt, the pupae were immediately placed in humid jars and incubated at 28°C. Flies emerged 20-26 days after larviposition. Six to 18 hours after emerging, the teneral flies were transferred into Geigy-1 fly cages which were then placed in a humidified room at 25°C and 75-80% relative humidity. Handling during the transfer to individual cages was facilitated by chilling the flies for up to five minutes in the freezer compartment of a domestic refrigerator.

### 3.11 Infection and Maintenance of Tsetse Flies

Teneral flies were fed within 24-36 hours of emergence on mice infected with T. congolense TREU 1690 or TREU 1682.. Control flies were fed on uninfected mice. The mice were individually restrained in the perforated barrel of a 60 ml plastic syringe on which a window covered with mosquito gauze had been cut for locating the Geigy-1 (Figure 3.3).

Flies were fed on mice which had been infected 2-8 days previously. The levels of parasitaemia varied between 1/20 field and 60-100/HPFs depending on the time after infection. Flies which did not feed within 36 hours of emergence were discarded.

All subsequent maintenance feeds were made at 2-3 day intervals on rabbits' ears (Plate 3.2). For each fly a record was made of its sex, number of days post-larviposition when it emerged, approximate age in hours, at which infected blood meal was taken and the days on which a maintenance feed was taken. Flies were tested for infectivity 21-28 days after feeding on the infected mice.

### 3.12 Identification of Infected Flies

The probe method for identifying infected tsetse flies (Burt, 1946b) could not be relied upon because it often took too long before a fly probed and metacyclics were easily washed from the slide when an attempt was made to fix and stain them. Since the two stocks used in the experiments were known to regularly produce local skin reactions in rabbits (Luckins and Gray, 1979) infected flies were identified by this method and later confirmed by dissection at the end of the experiments. Individual infected flies were fed in groups of 12-24 on the shaven backs of NZW rabbits, each fly being placed on an area marked out by picric acid. Positive reactions were confirmed by microscopical examination of chancre fluid for presence of trypanosomes.

### 3.13 Monitoring Skin Reactions

The development of local skin reactions was monitored by measurement of the diameters of hyperaemia and induration around the infective tsetse bite site. For each reaction, the diameter was measured in two or three directions and the mean value determined. Examination of the bite site and measurement of any visible reactions was usually carried out every day at 09.00-11.00 hours for up to 28 days for untreated rabbits or longer for rabbits which had been treated with trypanocidal drugs.

### 3.14 Histology of Skin

Material for histological examination was removed from rabbits which had been killed by cervical dislocation, skin containing the



local reaction, as well as skin on which uninfected flies had fed, was marked out with picric acid in living rabbits and then removed immediately after death. It was then trimmed, placed in Bouin's fixative for 24 hours, dehydrated through alcohol and finally embedded in paraffin wax. Sections 5  $\mu$ m thick were cut and stained using Giemsa colophonium (Appendix 2).

### 3.15 Estimation of Antibody Levels in Serum and Tissue Fluid

Determination of trypanosomal antibodies was made using an enzyme immunoassay and neutralization of infectivity test.

#### 3.15.1 Enzyme-linked immunosorbent assay (ELISA)

The ELISA test used was essentially that described by Silayo, Gray and Luckins (1980).

(a) Antigen preparation: Antigen was prepared from trypanosomes obtained from mice infected with T. congolense TREU 1690. Blood from heavily infected mice was applied to a column of DE52 and the separated trypanosomes were concentrated by centrifugation at 1500  $g$  and 4°C. Several trypanosome pellets were pooled and washed twice with 0.1M PBS pH 7.4 and stored at -20°C until used. For preparation of soluble antigen, the frozen trypanosome pellet was thawed, diluted 1/4 with ice-cold PBS, pH 7.4 and then subjected to 2 x 20 seconds ultrasonication at maximum amplitude on an MSE 100W ultrasonic disintegrator. During the sonication process the trypanosome suspension was jacketed in an ice-bath to prevent local overheating. Soluble antigen was separated from cell debris by ultracentrifugation at 4°C for 30 minutes at 100,000  $g$  in a Beckman L2-65B ultracentrifuge with SW 65L swing-out rotor. The protein concentration of the supernatant

was determined by ultraviolet spectrophotometry (Warburg and Christian, 1941) using a Pye Unicam SP 1800 Spectrophotometer. Protein concentrations of 100-300  $\mu\text{g/ml}$  were obtained. The soluble antigen preparation was diluted with PBS pH 7.4 and stored as a stock antigen at  $-20^{\circ}\text{C}$  in aliquots of 0.2-0.5 ml. For use in the test, stock antigen was diluted with 0.05M carbonate/bicarbonate buffer pH 9.6 to give a final protein concentration 1  $\mu\text{g/ml}$ .

(b) Immunoglobulin-enzyme conjugate: Goat anti-rabbit gamma-globulin conjugated with horse-radish peroxidase was obtained from Nordic Immunological Laboratories (Maidenhead, Berkshire, England). The freeze-dried conjugate was reconstituted with sterile distilled water and centrifuged at  $4^{\circ}\text{C}$  for 20 minutes at 1500  $g$  to remove insoluble material. It was then stored at  $-20^{\circ}\text{C}$  in aliquots of 50  $\mu\text{l}$  until used.

(c) Substrate: Orthophenylenediamine (OPD) at a concentration of 35 mg/100 ml phosphate citrate buffer pH 6.0, containing 0.03% hydrogen peroxidase (Analar, British Drug Houses) was used as the substrate.

(d) Procedure for the microELISA test: MicroELISA plates (Cooke microELISA M29 AR) were coated with trypanosomal antigen by placing 250  $\mu\text{l}$  of diluted antigen solution in each well and incubating the plates overnight at  $4^{\circ}\text{C}$ . Plates were sealed with Scotch pressure sensitive tape. Unadsorbed antigen was washed out by emptying the wells and filling them with PBS pH 7.4 containing 0.05% Tween 20 (PBS/Tween 20) three times for three minutes. The plates were then shaken dry and each well filled with 250  $\mu\text{l}$  of serum or tissue fluid sample

diluted 1/1000 with PBS/Tween 20. Each sample was assayed in triplicate on the same plate. Controls included PBS/Tween 20, serum or tissue fluid from uninfected rabbits and serum or tissue fluid from a known infected rabbit collected 42 days after infection with T. congolense TREU 1690. The plates were sealed, wrapped in aluminium foil and incubated at 37°C on top of a water-bath for 15 minutes. Washing was repeated to remove unreacted antibody and then 250 µl of horse-radish peroxidase immunoglobulin conjugate diluted 1/1000 with PBS/Tween 20 was added and incubated for 15 minutes at 37°C. After washing and drying the plates, 250 µl of substrate at 4°C was added to each well. The plate was sealed and wrapped in foil and then incubated at 37°C for 30 minutes. The enzyme reaction was stopped by adding 100 µl of 1M "Aristar" sulphuric acid (British Drug Houses). The absorbances of the solutions in each well were measured at 492 nm in SP 200 Pye Unicam Spectrophotometer or an MSE/Fisons VITATRON Universal photometer adapted for microELISA.

The mean readings for the triplicate assay carried out for each sample on the same plate were standardised according to the following formula:-

$$\text{Corrected ELISA value} = (U_n - N_n) \times \frac{P_o - N_o}{P_n - N_n}$$

where  $U_n$  = ELISA value of unknown test sample on day  $n$

$N_n$  = ELISA value of normal serum on day  $n$

$P_o$  = ELISA value of positive serum control on initial day of series of assays

$N_o$  = ELISA value of normal serum control on initial day of assays

$P_n$  = ELISA value of positive control serum on day  $n$

### 3.15.2 Neutralization of infectivity test (NIT)

Neutralization tests were carried out by incubating trypanosomes in diluted serum or tissue fluid and then determining the infectivity of the suspension by mouse subinoculation. Trypanosomes were derived from T. congolense TREU 1725 by two subpassages at 4-day intervals in mice immunosuppressed with 300 mg/kg cyclophosphamide.

Trypanosomes from infected, immunosuppressed mice were separated from blood elements by elution through DE52 cellulose column and concentrated by centrifugation at 4°C for 15 minutes at 1500 g. The resulting trypanosome pellet was resuspended in a small amount of PBSG with 10% fetal bovine serum (PBSG/FBS) and the trypanosome concentration estimated by counting the number of parasites in an improved Neubauer hemocytometer (Lumsden et al., 1973). It was then diluted with more PBSG/FBS so as to contain 250,000 trypanosomes/ml. Test samples were diluted 1/10 by mixing together 0.2 ml of serum or tissue fluid, 1.6 ml of PBSG/FBS and 0.2 ml of trypanosome suspension. This suspension was incubated for 30 minutes at 4°C and then 0.2 ml amounts inoculated intraperitoneally into groups of five mice. Control normal serum was also assayed. The mice were examined twice a week for 35 days or until they became positive.

Figure 3.1

Derivation of stabilates prepared from Trypanosoma congolense stock (ZARIA/67/LUMP/79).

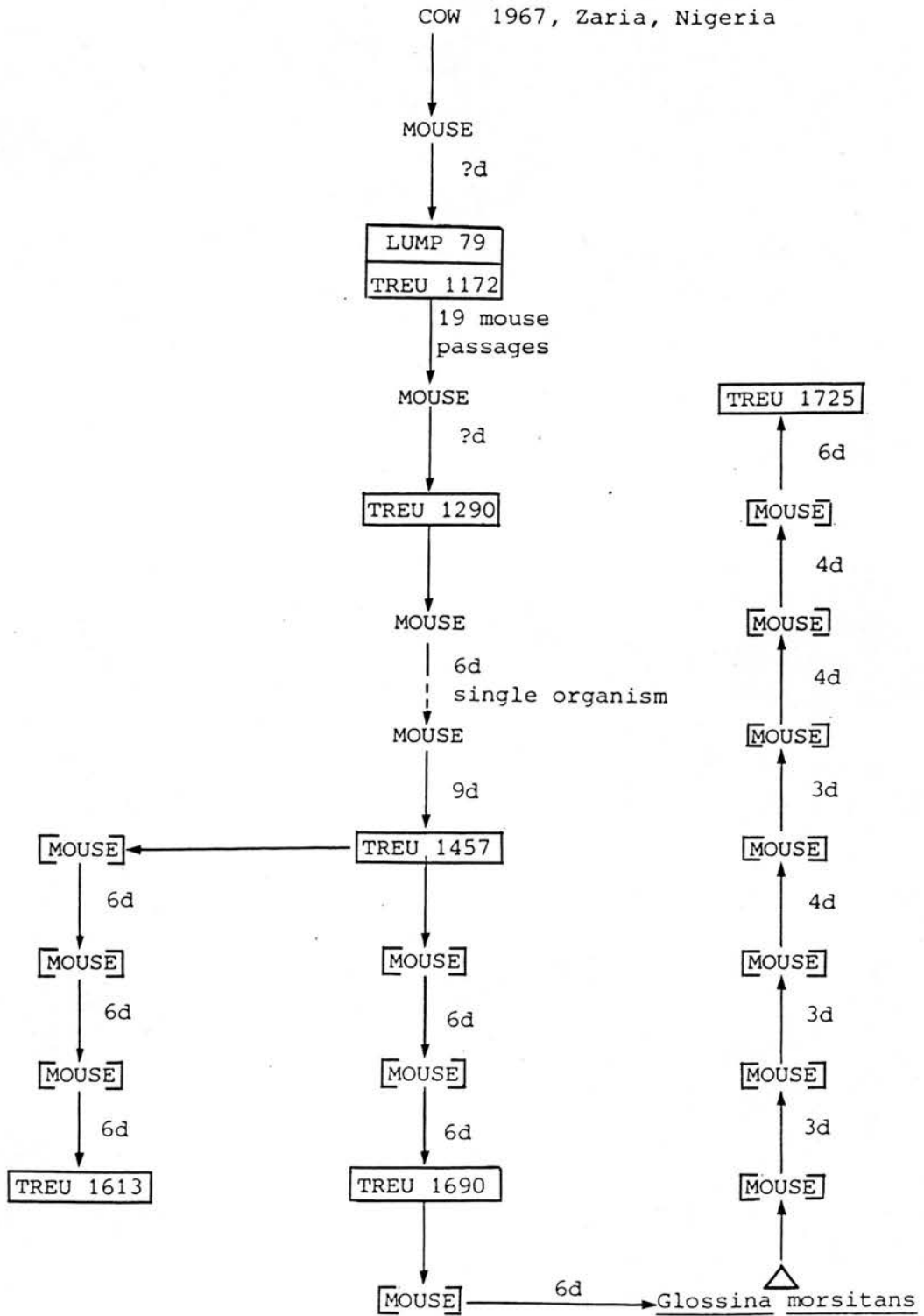


Figure 3.2

Derivation of stabilates prepared from Trypanosoma congolense stock (SERENGETI/66/LUMP/12).

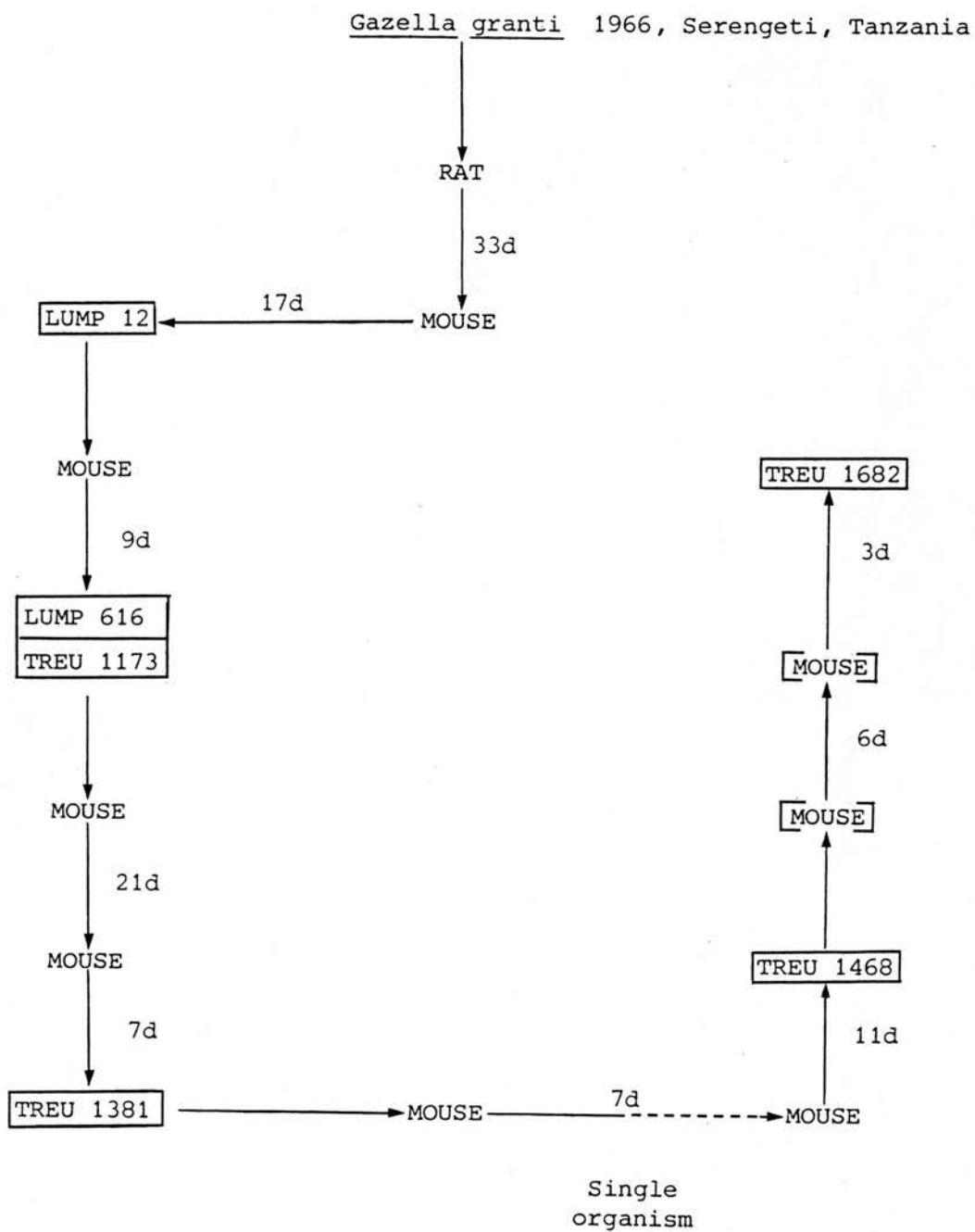


Plate 3.1A Thin smear of mouse blood obtained at day 15  
post-infection with T. congolense TREU 1457  
(Giemsa, x 625)

Plate 3.1B Thin smear of cultured metacylics of T. congo-  
lense TREU 1457.  
(Giemsa, x 625)





Figure 3.3 Plastic syringe (60 ml) modified for restraining a mouse to allow feeding of a tsetse-fly placed in Geigy-1 cage.

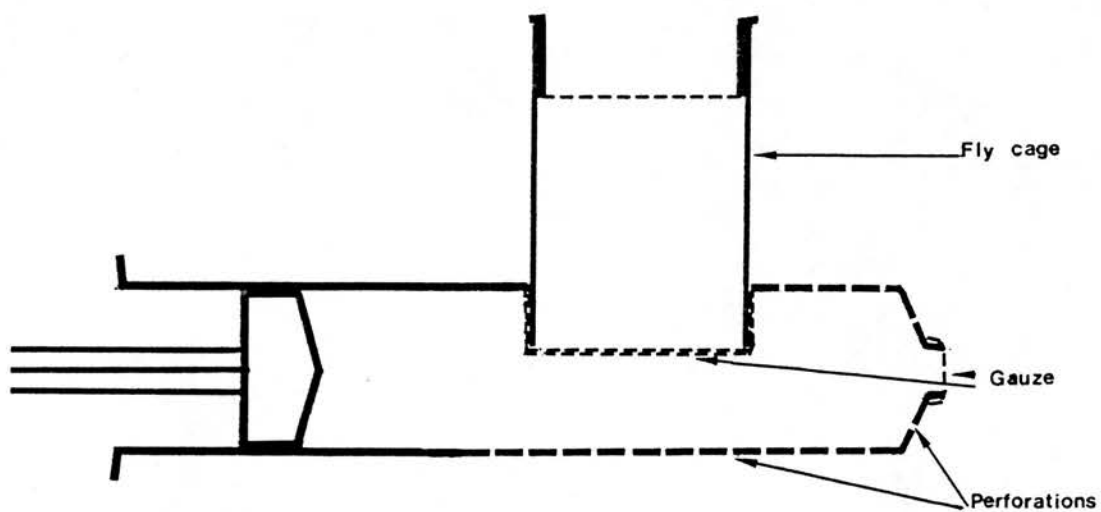


Plate 3.2 Feeding tsetse flies in Geigy-1 cages on ears of  
Sandylop rabbits.



## CHAPTER FOUR

## INFECTIVITY, PATHOGENICITY AND DRUG SENSITIVITIES

4.1 Introduction

T. congolense infects most species of domestic mammals but there are variations in infectivity and pathogenicity of different stocks (Hoare, 1972). It is generally considered that West African stocks of T. congolense are of lower pathogenicity to cattle than East African stocks (Stephen, 1970; Losos and Ikede, 1972). In studies on West African isolates of T. congolense, pathogenicity was found to correlate with trypanosome morphology and dimensions (Godfrey, 1961). Short congolense type trypanosomes were characterised by low infectivity, low parasitaemia and low pathogenicity, while long dimorphon types had high infectivity, high parasitaemia and high pathogenicity. Intermediate stocks were characterised by high infectivity and high parasitaemias but low pathogenicity.

The infectivity and pathogenicity of stocks of T. congolense to mice, rats, guinea pigs and rabbits is generally low on initial isolation. Many stocks obtained from the field cannot be isolated in laboratory animals (Godfrey, 1982). However, the infectivity and pathogenicity of an isolate can be enhanced upon serial passage (Losos and Ikede, 1972). The resulting infection changes in certain important characteristics, including drug sensitivity. Thus, results obtained in experimental work with laboratory rodents may not necessarily be applicable to natural infections in livestock. This is particularly the case with infections in mice, rats and guinea pigs where high parasitaemias invariably leading to death, commonly occur.

Among laboratory animals the rabbit is probably the most suitable host for studies on T. congolense since low parasitaemias are obtained and the early events after cyclical infection with the parasite have been shown to be similar to those occurring in cattle (Gray and Luckins, 1980).

The pathogenicity of T. congolense in rabbits and cattle has also been compared (Holmes, 1976). The mean packed cell volume (PCV) of the infected rabbits was observed to drop from a normal of 40% to 28% while in cattle the PCV drop was from a normal of 30% to 14%. Both species showed a normochromic, normocytic anaemia but whereas the disease in cattle consistently led to death at 5-10 weeks after infection there was rarely any mortality among rabbits.

In contrast, severe disease has been reported in rabbits inoculated with  $5 \times 10^6$  bloodstream forms of T. congolense (Mansfield and Kreier, 1972a). Lesions normally associated with T. brucei infections in rabbits were seen and death always occurred unless treatment with homidium was carried out. Even then, few or no trypanosomes were observed microscopically although the blood was infective to rats.

For reasons of economy, mice are used for studies on drug sensitivities of T. congolense stocks, although there is the probability that trypanosome isolates may change their drug sensitivity upon passage. A technique for determining anti-trypanosomal activity of new compounds in mice, described by Hawking (1963a) can also be applied to investigations on the sensitivity of trypanosome stocks to the commonly used trypanocides. Treatment is carried out when the mice show a parasitaemia of 0.1-10 trypanosomes/HPF. It is necessary to standardise the infecting dose so as to obtain uniform parasitaemias

at the time of treatment, since it has been shown that effective and curative doses are proportional to the numbers of trypanosomes used in infecting the mice (Raether, 1970; Walker and Opiyo, 1973).

Trypanosome drug sensitivity in vivo is usually expressed in terms of the minimum effective dose (MED) or the minimum curative dose (MCD) for a particular animal species. The MED is the dose which clears the trypanosomes from 80% of the animals treated ( $ED_{80}$ ) while the MCD is the dose which cures 80% of treated animals ( $CD_{80}$ ) (Hawking, 1963b).

In the following experiments, preliminary studies of drug sensitivity were carried out on two stocks of T. congolense later used for studies on the effect of drug treatment on the development of sterile immunity to T. congolense infection in rabbits. The two stocks were tested in mice for their sensitivity to diminazene aceturate and homidium bromide and in rabbits for sensitivity to diminazene acetate and isometamidium chloride.

Observations were also made on the possible influence of the levels and duration of parasitaemia in mice on which tsetse flies were fed, and the sex of the fly on trypanosome infection rates in the flies.

The gross appearance and reproducibility of the local skin reactions following bites by infected tsetse flies were studied in rats, guinea pigs and rabbits and the course of infection in the rabbit was also monitored.

## 4.2 Drug Sensitivity of Trypanosoma congolense stocks

### 4.2.1 In mice

Experimental design: Two stocks of T. congolense, TREU 1468 and TREU 1613 were tested for their sensitivity to diminazene aceturate and homidium bromide. The drug assay was carried out as described by Hawking (1963a). A batch of CF1 mice 6-8 weeks old and weighing 20-25 g were divided into groups which were inoculated intraperitoneally with approximately  $10^4$  T. congolense bloodstream forms obtained from mice. Mice inoculated with each stock were then randomly allocated in groups of five and marked with picric acid to identify the individual mouse, the group it belonged to and the drug to be used for treatment. They were then examined daily for the presence of parasites in tail blood and at 4-6 days post-infection when the parasitaemia was 0.1-4 parasites per HPF, the groups were again randomly allocated for treatment at different dosage levels of the drugs. Drugs were prepared fresh on the day of treatment and were inoculated intraperitoneally. Parasitaemia was monitored by wet smear examination for up to seven days after treatment and thereafter twice weekly up to 35 days post-treatment.

Results: Tables 4.1 and 4.2 show the numbers of mice infected with T. congolense TREU 1468 or TREU 1613 after treatment at different dose levels with homidium bromide or diminazene aceturate. Mice in all test groups were positive on the day of treatment (day 0). By day 3 after treatment, trypanosomes had disappeared from some of the treated mice, but reappeared by day 30. From the numbers of mice parasitaemic at days 3 and 30 after treatment, the minimum curative doses can be estimated. These are shown in Table 4.3. T. congolense



TREU 1468 appeared to be more sensitive than T. congolense TREU 1613 to both homidium bromide and diminazene aceturate. For example, the MCD of diminazene aceturate for T. congolense TREU 1468 was 10 mg/kg compared to 20 mg/kg for T. congolense TREU 1613.

#### 4.2.2 In rabbits

Experimental design: The MCD value of 20 mg/kg diminazene aceturate for mice infected with T. congolense TREU 1613 was used as a guide in calculating the approximate MCD in rabbits, using the dose/surface area relationship whereby dose is proportional to two-thirds the power of the body weight (Moore, 1909; Bushby, 1963). The calculated MCD of diminazene aceturate for the rabbit was 4.3 mg/kg.

To determine the practical MCD in rabbits experiments were done using animals infected with T. congolense TREU 1457 from which TREU 1613 was derived. Three pairs of rabbits, which had been infected by G. m. morsitans 24-66 days previously were treated with diminazene aceturate at dose rates of 3.5, 4.0 and 5.0 mg/kg. The presence of trypanosomes in the blood on the day of treatment was confirmed by the microhaematocrit/buffy coat technique (Murray et al., 1977). After treatment, blood was checked for trypanosomes twice a week by the microhaematocrit/buffy coat technique until the animals became positive again or for up to 42 days. At this time, blood was subinoculated into mice to confirm the absence of trypanosomes. Animals which were negative for trypanosomes in the blood for a period of 42 days after treatment were considered to be cured. In another experiment the curative dose of isometamidium chloride for T. congolense TREU 1690 infection in the rabbit was determined. Four rabbits which had been infected by bites from G. m. morsitans 21 days previously were

treated with isometamidium chloride at dose rates of 0.25, 0.50, 0.75 and 1.0 mg/kg. Determination of presence of trypanosomes on the day of treatment and after treatment was carried out as described previously.

Results: In the rabbits treated with diminazene aceturate, 5.0 mg/kg dose rate was completely curative while 3.5 mg/kg and 4.0 mg/kg were both only partially curative (Table 4.4). Thus the MCD for T. congolense TREU 1457 is between 4.0 and 5.0 mg/kg.

In the animals treated with isometamidium chloride it was found that treatment at 0.25 or 0.50 mg/kg dose rate resulted in relapses in parasitaemia after treatment while there was no relapsed parasitaemia after treatment at 0.75 mg/kg or 1.0 mg/kg dose rates (Table 4.5).

#### 4.3 Infectivity of Trypanosoma congolense TREU 1690 for Glossina morsitans morsitans

Experimental design: Teneral flies were fed individually on mice infected with T. congolense TREU 1690 within 36 hours of emergence. Flies were offered a blood meal 2-3 times within approximately 6-18 hours of emergence, until they fed. The mice on which the teneral flies took their first blood meal had infections of 2-8 days duration. The parasitaemia was not quantitated daily but was of the order of  $10^5$ - $10^6$  trypanosomes/ml at 1-4 days and  $10^6$ - $10^8$  at 5-8 days as estimated by the rapid "matching" method of Herbert and Lumsden (1976) applied to tail blood.

Flies were tested for infectivity at day 21-24 after emerging, by being fed on the shaved backs of susceptible rabbits to induce chancres. Flies whose bites did not induce chancres within 7-8 days

of feeding were then dissected. Flies which induced chancres were dissected at the end of experiments.

The flies known to be infected were maintained on normal rabbits or on mice for testing maintenance of infectivity. Batches of infected flies were not maintained on the same rabbit for more than 14 days after the first infected fly fed.

The final fly infection rates were obtained by combining the results from fly dissection and from observations on chancre induction.

Results: One hundred and eleven flies out of 174 (64%) were positive, with infection of mouthparts. Table 4.6 compares the number of infected and non-infected male and female flies fed on mice with infections of 1-4 days or 5-8 days duration. There was no statistically significant difference in infection rates between these two groups. The infection rates were also analysed for the influence of sex of fly (Table 4.7). There was no significant difference between infection rates in male and female flies.

#### 4.4 Gross Appearance and Reproducibility of the Local Skin Reaction to Trypanosoma congolense

Experimental design: In preliminary studies, rats and guinea pigs were examined for development of chancres at skin sites bitten by G. m. morsitans infected with T. congolense TREU 1457. Up to four infected flies were allowed to feed on shaved areas of each of five guinea pigs and five rats. The animals were then checked for chancre development every day for the first 14 days and three times a week thereafter for up to 28 days after infection. They were also examined three times a week for the presence of trypanosomes in blood

from ear vein (guinea pig) or tail (rat).

Studies were also carried out to determine the reproducibility of the induction of chancres in rabbits by tsetse flies infected with T. congolense. Fourteen G. m. morsitans infected with T. congolense TREU 1457 and five non-infected flies were fed on a number of different occasions on shaved areas on the backs of 13 normal rabbits. The diameter of the erythematous and indurated area around the bite sites was monitored daily for up to 21 days after infection. The majority of rabbits were observed for up to 12 days after infection, when they were killed for histological studies.

Results: No skin reaction appeared on any of the guinea pigs or rats. All the guinea pigs were parasitaemic within 21-28 days after infection while the rats had become parasitaemic 7-15 days after infection.

In contrast, local skin reactions appeared in rabbits at sites where infected flies had fed. Table 4.8 compares the frequency of occurrence of local skin reactions  $\geq 5$  mm in diameter at sites bitten by infected or uninfected flies. Forty-seven out of 55 (85%) bites from infected flies gave reactions  $\geq 5$  mm diameter whereas only four out of 35 (11%) bites from non-infected flies gave reactions  $\geq 5$  mm diameter. The reactions from non-infected flies were of short duration (2-3 days) and reached a maximum of less than 10 mm. In contrast, the reactions from infected flies reached a maximum diameter of 50 mm and lasted up to 11 days. Plates 4.1-4.3 show the skin reactions at different times after infective tsetse bites on the shaved backs of two of the rabbits.

#### 4.5 The Course of Infection in Rabbits Infected with Trypanosoma congolense by Glossina morsitans morsitans

Experimental design: Rabbits which had been cyclically infected with T. congolense TREU 1690 and then treated with diminazene aceturate were monitored for changes in packed cell volume, weight and antibody levels up to 12 weeks after infection.

Ten rabbits approximately 16 weeks old, were randomly allocated into three groups. One group of four animals was then used for experimental infection and treatment while the other two groups were used as uninfected, untreated and uninfected, treated control groups. Each animal in the experimental group was infected by bites from four tsetse flies infected with T. congolense TREU 1690. The experimental and uninfected treatment control groups were treated with 14 mg/kg diminazene aceturate at seven weeks after infection of the experimental group.

All animals were weighed and bled for serum and blood once a week, from one week before infection to 12 weeks after infection. For PCV measurements three heparinised capillary haematocrit tubes (Heparinised Benjamin Haematocrit tubes - Harshaw Chemicals Ltd., England) were filled with blood direct from the punctured ear vein and then sealed with vinyl plastic putty (Cristoseal, Lancer St., Louis, U.S.A.). The sealed capillary tubes were centrifuged for four minutes on a microhaematocrit centrifuge (Hawksley, England) and the PCV measured on a Hawksley Microhaematocrit Reader.

The infected experimental group was monitored for chancre development up to 21 days after infective tsetse bite and checked for antibody levels in serum, using the ELISA test. Development of parasitaemia

was monitored once a week by the microhaematocrit/buffy coat technique from two weeks to 12 weeks after infection. Absence of trypanosomes in blood after treatment was confirmed by the inoculation of 0.5 ml amounts of blood into each of three mice at the end of the experiment.

Results: Mean PCV of infected, treated group dropped from a pre-infection level of 40% to 30%, six weeks after infection, then rapidly returned to normal one week after treatment (Figure 4.1 and Appendix 3). Trypanosomes were detectable by the microhaematocrit/buffy coat technique throughout the period between infection and treatment. After treatment, no trypanosomes could be detected by the microhaematocrit/buffy coat technique or mouse subinoculation. The control uninfected groups maintained mean PCVs of above 40% throughout the period of observation.

The T. congolense infection did not appear to affect growth rate; the mean weights of experimental and treatment control groups were virtually the same throughout (Figure 4.2 and Appendix 4). The uninfected, untreated control group showed mean weight loss between weeks 7 and 10.

Chancre diameters and anti-trypanosomal antibody levels as measured by ELISA are shown in Figure 4.3. Peak chancre sizes were attained on day 11 except for one rabbit (1069) in which the peak appeared earlier. All infected rabbits had detectable antibody levels from week 2 after infection. There was a gradual fall in antibody levels after drug treatment.

Table 4.1

Sensitivity of Trypanosoma congolense TREU 1468 and TREU 1613 to treatment with different doses of HOMIDIUM BROMIDE in mice.

Stock	Days after treatment	Drug Dose (mg/kg)				
		Number of mice infected		Number treated		
		Untreated control	0.02	0.1	0.5	2.5
TREU 1468	0	5/5	5/5	5/5	5/5	5/5
	3	5/5	4/5	0/5	0/5	0/5
	30	5/5	5/5	5/5	4/5	0/5
TREU 1613	0	5/5	5/5	5/5	5/5	5/5
	3	5/5	5/5	5/5	5/5	2/5
	30	5/5	5/5	5/5	5/5	5/5

Table 4.2

Sensitivity of Trypanosome congolense TREU 1468 and TREU 1613 to treatment with different doses of DIMINAZENE ACETURATE in mice.

Stock	Days after treatment	Drug dose (mg/kg)				
		Untreated control	Number of mice infected	Number treated		
			1.25	2.5	5.0	20.0
TREU 1468	0	5/5	5/5	5/5	5/5	5/5
	3	5/5	1/5	0/5	0/5	0/5
	30	5/5	5/5	5/5	4/5	1/5
TREU 1613	0	5/5	5/5	5/5	5/5	5/5
	3	5/5	5/5	0/5	0/5	0/5
	30	5/5	5/5	5/5	5/5	1/5



Table 4.3

Sensitivities of *Trypanosoma congolense* TREU 1468 and TREU 1613 to treatment with HOMIDIUM BROMIDE and DIMINAZENE ACETURATE in mice: Estimated minimum effective dose (MED) and minimum curative dose (MCD).

Stock	Drug Dose (mg/kg)			
	Homidium bromide		Diminazene aceturate	
	MED	MCD	MED	MCD
TREU 1468	0.1	2.5	1.25	10.0
TREU 1613	2.5	>12.5	2.5	20.0

Table 4.4

Sensitivity of Trypanosoma congolense TREU 1457 to treatment with different doses of DIMINAZENE ACETURATE in the rabbit.

Rabbit Number	Days post-infection when treated	Dose (mg/kg)	Presence of Trypanosomes	
			Pre-treatment	Post-treatment*
821	66	3.5	+	0
866	24	3.5	+	+
816	34	4.0	+	+
858	38	4.0	+	0
815	34	5.0	+	0
861	31	5.0	+	0

\*0 = No trypanosomes detected by mouse subinoculation

Table 4.5

Sensitivity of Trypanosoma congolense TREU 1690 to treatment with different doses of ISOMETAMIDIUM CHLORIDE in the rabbit.

Rabbit Number	Days post-infection when treated	Dose (mg/kg)	Presence of Trypanosomes	
			Pre-treatment	Post-treatment
1124	21	0.25	+	+
1120	21	0.50	+	+
1122	21	0.75	+	0
1123	21	1.00	+	0

\*0 = No trypanosomes detected by mouse subinoculation

Table 4.6

Comparison of numbers of infected and non-infected Glossina morsitans morsitans fed on mice 1-4 days or 5-8 days after infection with Trypanosoma congolense TREU 1690.

A. Female G. m. morsitans

Status	Duration of Infection in Mice		$\chi^2$	Assessment
	1-4d	5-8d		
Infected	17	32	1.560	Not significant
Non-infected	16	17		

B. Male G. m. morsitans

Status	Duration of Infection in Mice		$\chi^2$	Assessment
	1-4d	5-8d		
Infected	48	14	0.006	Not significant
Non-infected	23	7		

Table 4.7

Comparison of numbers of infected and non-infected female and male Glossina morsitans morsitans fed on mice 1-4 days or 5-8 days after infection with Trypanosoma congolense TREU 1690.

A. Flies fed on mice with infection of 1-4 days duration

Status	Sex of Fly		$\chi^2$	Assessment
	Females	Males		
Infected	17	48	2.489	Not significant
Non-infected	16	23		

B. Flies fed on mice with infection of 5-8 days duration

Status	Sex of Fly		$\chi^2$	Assessment
	Females	Males		
Infected	32	14	0.012	Not significant
Non-infected	17	7		

Table 4.8

Maximum diameters of local skin reactions at sites bitten by infected and non-infected Glossina morsitans

Rabbit Number	Maximum Diameter in Millimetres			
	Flies infected with <u>T. congolense</u>		Non-infected flies	
	>5	≤5	>5	≤5
815	2	0	0	1
816	3	0	1	1
817	5	0	2	1
821	1	0	0	0
823	4	0	0	2
825	4	1	0	3
826	3	1	0	2
829	3	0	0	5
830	3	0	0	5
831	3	0	1	4
855	7	3	0	4
858	3	0	0	2
861	6	3	0	1
Total	47	8	4	31

Plate 4.1A Chancres on rabbit 823 at day 7 after tsetse bite.

Plate 4.1B Chancres on rabbit 823 on day 10 after tsetse bite.  
Note greyish white zones, also described in cattle  
(Akol and Murray, 1982).

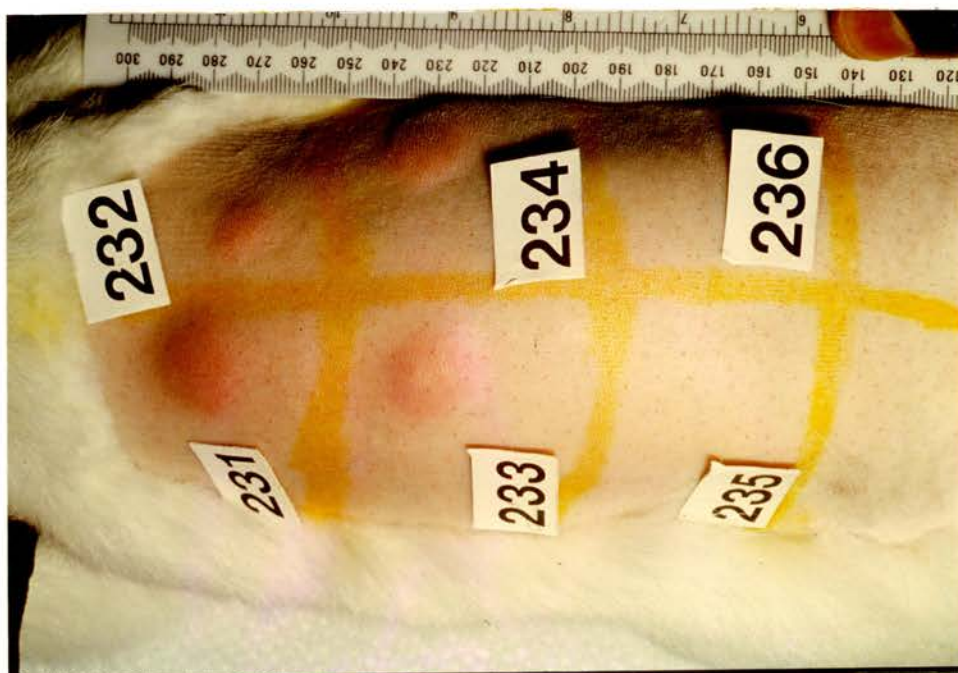




Plate 4.2A Chancres on rabbit 831 at day 8 after tsetse bite.

Plate 4.2B Chancres on rabbit 831 on day 11 after tsetse bite. Note severe reactions characterised by depressed red centres with crust formation on two chancres (top left and centre bottom) and also presence of a late developing chancre (top right hand corner).

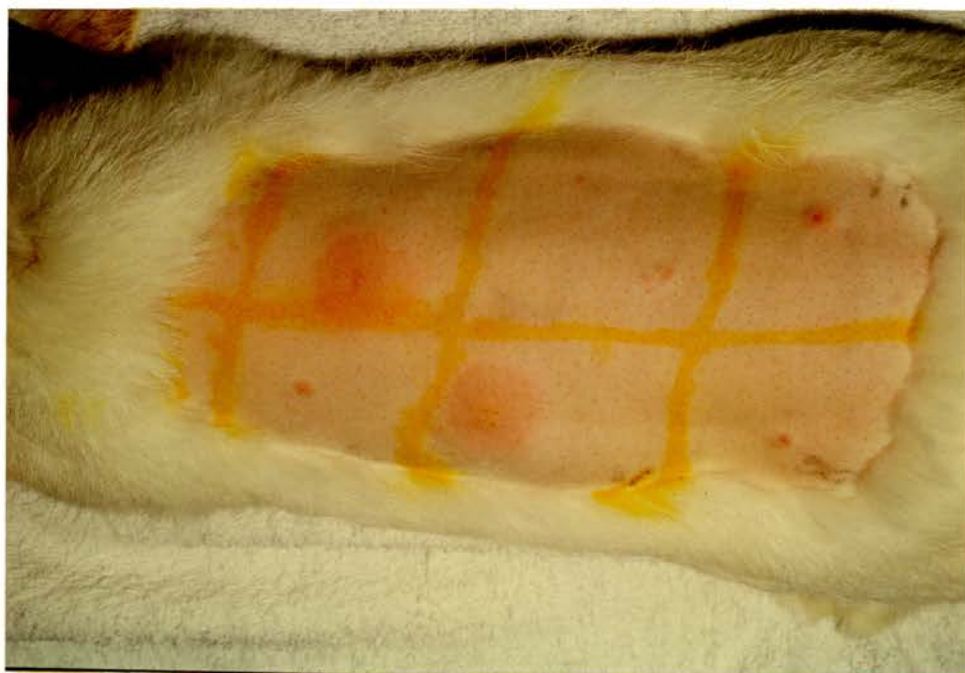


Plate 4.3A Chancres on rabbit 831 on day 14 after tsetse bite. Note late developing chancre has still not formed a greyish white or red-encrusting centre, while two earlier developing chancres are disappearing and are only discernible as red encrusting areas.

Plate 4.3B Rabbit 831 on day 17 after tsetse bite. Chancres have completely disappeared, including the late developing skin reaction which was in the top right hand corner.

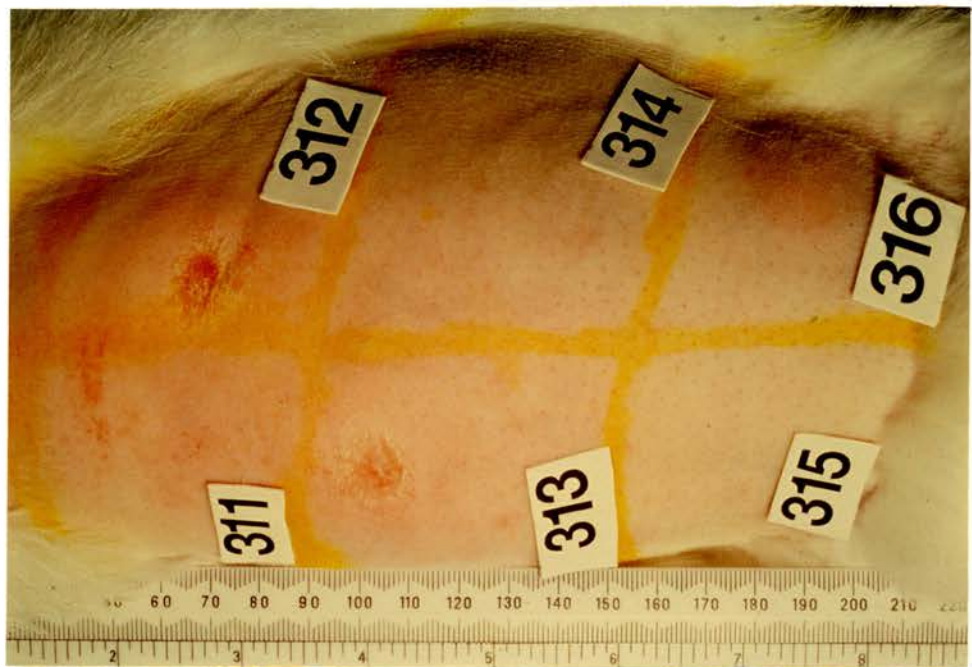


Figure 4.1 Packed cell volumes (PCVs) of rabbits cyclically infected with Trypanosoma congolense TREU 1690.

Key

- Infected, treated group (Rabbits 1069, 1073, 1075 and 1077).
- Uninfected, treated group (Rabbits 1067, 1070 and 1071).
- ▲ Uninfected and untreated group (Rabbits 1072, 1074 and 1076).
- B** ▼ Treatment with diminazene aceturate (Berenil)

FIG. 4.1

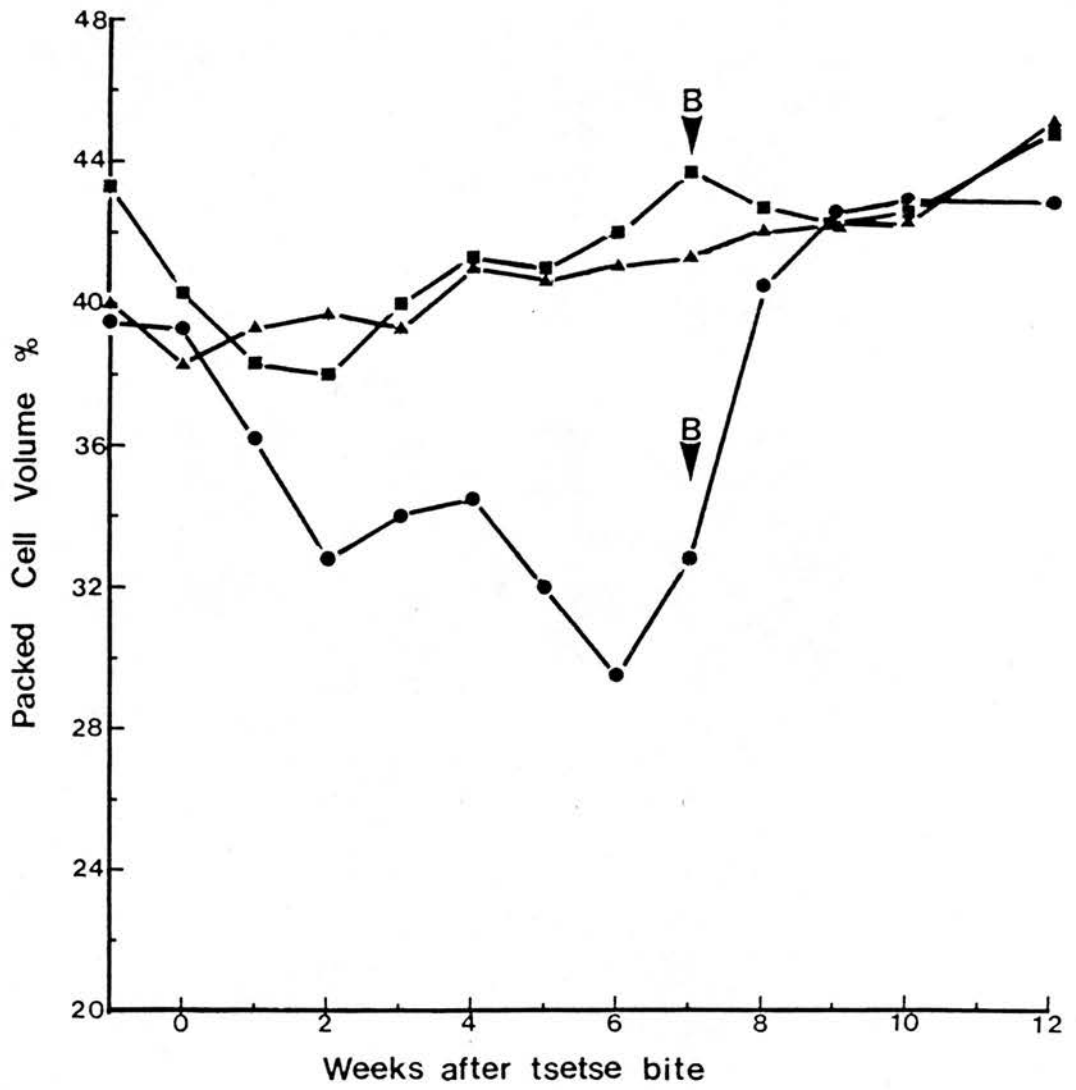
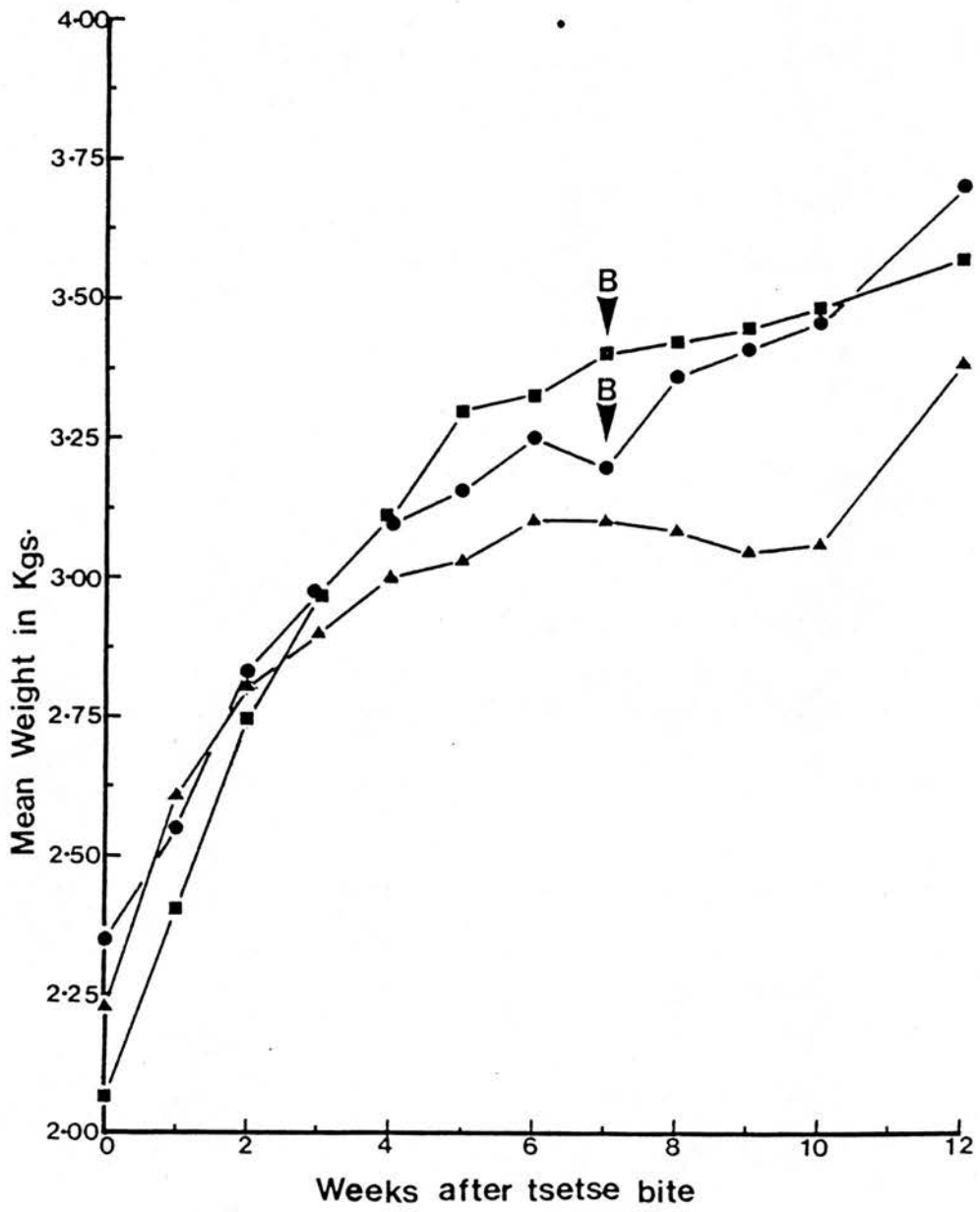


Figure 4.2 Mean body weights of uninfected rabbits and of rabbits cyclically infected with Trypanosoma congolense TREU 1690.

Key as in Figure 4.1

FIG. 4.2



Figures 4.3 A-C Mean diameters of chancres and mean ELISA values ( $E_{492}$ ) of serum anti-trypanosome antibodies in rabbits infected with T. congolense TREU 1690 and then treated with diminazene aceturate.

Key

● Rabbit 1069

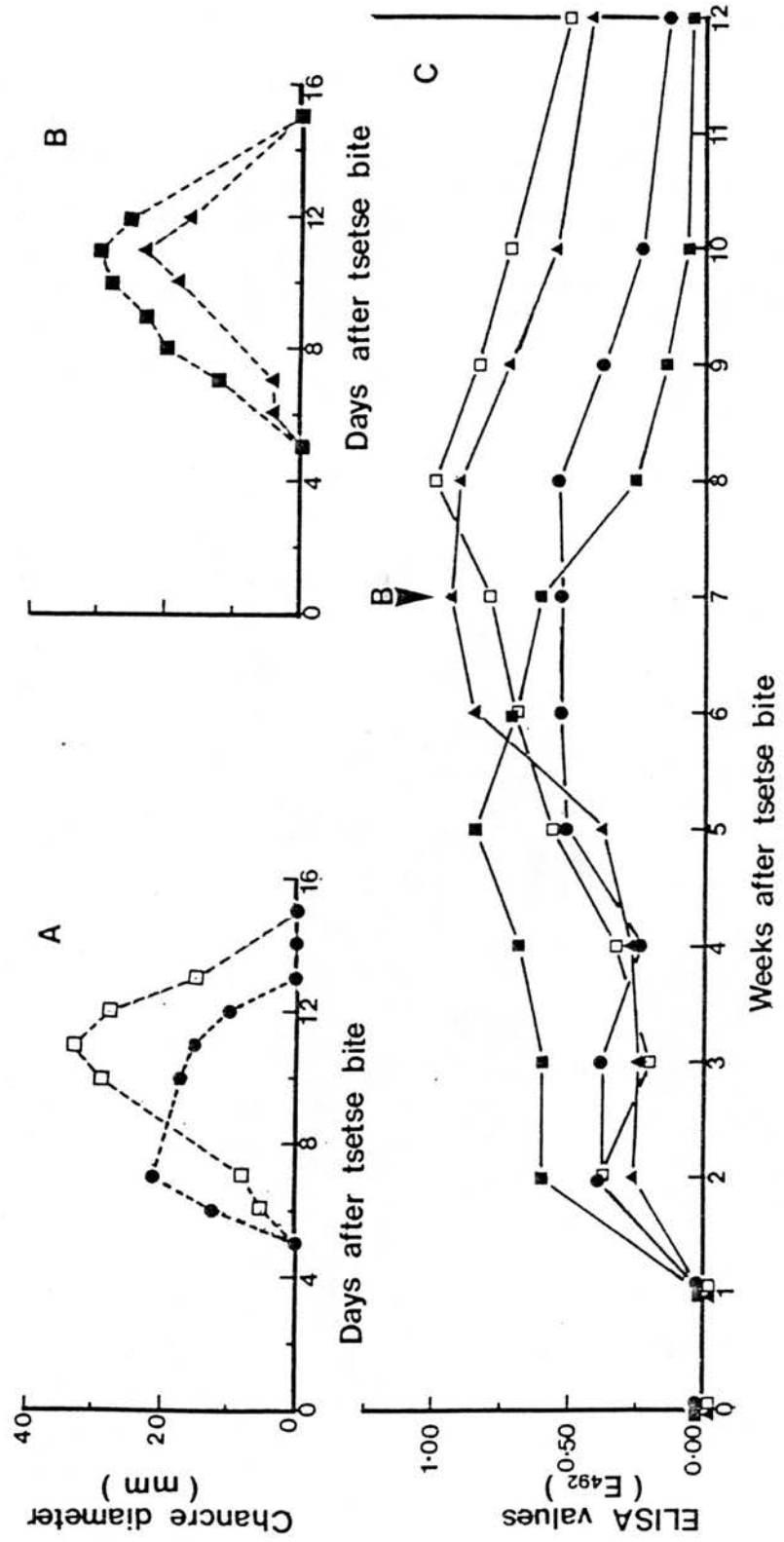
■ Rabbit 1073

▲ Rabbit 1075

□ Rabbit 1077

**B** Treatment with diminazene aceturate  
▼ (Berenil)

FIG. 4.3



#### 4.6 Discussion

The sensitivity of the two stocks of T. congolense to treatment with diminazene aceturate (MCD 10 mg/kg and 20 mg/kg for TREU 1468 and TREU 1613 respectively) were within the range for drug sensitive stocks found in other studies. Hawking (1963b) reported MCD ranges of 10-15 mg/kg for drug sensitive stocks of T. congolense whereas T. congolense stocks which were resistant to diminazene aceturate and/or quinapyramine, prothidium and metamidium had MCDs of 25-100 mg/kg. Moreover, T. congolense stocks TREU 1468 and TREU 1613 had MEDs 1-5 mg/kg which are within the range of MEDs of the T. congolense stocks considered by Hawking (1963b) as sensitive to diminazene aceturate.

Differences in levels of parasitaemia at the time of drug treatment of mice could account for the lower minimum curative dose of T. congolense TREU 1468 compared to TREU 1613 found in this study. Although mice had been inoculated with approximately equal numbers of T. congolense TREU 1468 or TREU 1613, the groups inoculated with T. congolense TREU 1613 always had higher levels of parasitaemia at the time of treatment. Other studies have shown that MCDs and MEDs in mice are directly proportional to parasitaemic level at the time of treatment (Raether, 1970; Walker and Opiyo, 1973).

The MCD value of diminazene aceturate for T. congolense TREU 1613 was used to estimate the MCD for the rabbit by calculations based on a dose-surface area relationship formula (Moore, 1909; Bushby, 1963). A probing experiment to determine the practical MCD of diminazene aceturate for rabbits infected with T. congolense TREU 1690 showed that 5.0 mg/kg was curative while 3.5 mg/kg and 4.0 mg/kg were only partially curative. Diminazene aceturate at 3.5 mg/kg has previously been shown

to be subcurative for rabbits infected with a related stock of T. congolense (Gilbert and Newton, 1982b; Gilbert, 1983).

The recommended dose of diminazene aceturate in cattle is 3.5 mg/kg (Williamson, 1970; Anon, 1979). From this, an equivalent dose-surface area-calculated therapeutic level of diminazene aceturate for rabbits would be approximately 16 mg/kg. However, the recommended dose for bovine trypanosomiasis is intentionally made far higher than the MCD for sensitive stocks, as a safety measure against, for example underestimation of animal body weight as well as possible occurrence of slightly drug resistant stocks. Initial studies indicated that 1 mg/kg diminazene aceturate was curative for T. congolense infection in cattle (Bauer, 1955). It was decided that in experiments in which efficacy of diminazene aceturate against cyclical T. congolense infection was being studied, a minimum dose of 7 mg/kg would be used, but higher doses would be applied where the main objective was to abrogate the infection for other studies such as immunity.

The number of animals used for testing sensitivity of T. congolense TREU 1690 to isometamidium chloride was too low to allow estimation of the minimum curative dose. However, the result obtained indicates the MCD was between 0.50 mg/kg and 1.0 mg/kg. Thus it was decided that for prophylaxis purposes, 4 mg/kg dose rate would be used.

With regard to T. congolense infection rates in G. m. morsitans it was shown that high infection rates of up to 64% could be obtained. This is in contrast to the low infection rates of 8-14% reported by others in studies in which G. m. morsitans were offered infective blood meals and maintained on mice, rabbits and goats (Nantulya et al., 1978b;

Luckins and Gray, 1979a; Moloq, 1981). High T. congolense infection rates of 46% have been reported in G. m. morsitans maintained on goats (ILRAD, 1983).

It is interesting that no significant differences were found in infection rates of flies fed on mice with 5-8 day infections as opposed to mice with 1-4 day infections. Mice fed upon on days 1-4 after infection had parasitaemias of  $10^5$ - $10^6$  trypanosomes/ml while those fed upon on days 5-8 post-infection, had parasitaemias of  $10^6$ - $10^8$  trypanosomes/ml. This appears to confirm earlier findings in which the concentration of trypanosomes in the infected blood meal was not found to influence infection rates (Baker and Robertson, 1957; Wijers and Willett, 1960; Dipeolu, 1975). It is however in contrast to the findings of Nantulya et al. (1978b) who reported higher T. congolense-infection rates (22%) in G. m. morsitans fed on mice with peak parasitaemia than at log phase or "falling phase" of parasitaemia (7% in each case). Although in the studies of Nantulya et al. (1978b) much larger numbers of flies were used, it is also probable that the difference is due to the use of a different technique of infecting and maintaining the tsetse flies.

The finding that there was no significant difference between infection rates of male and female tsetse flies confirms previous findings (Baker and Robertson, 1957; Dipeolu, 1972).

In the present work, flies which did not feed within 36 hours of emergence were discarded because the earlier studies indicated that mature flies do not become infected (Wijers, 1958). Recent studies with T. brucei organisms have indicated that flies of up to five days of age at first blood meal can be infected (Gingrich et al.,

1982). There are also reports that tsetse flies can become infected with T. congolense when their first infected blood meal is taken after the teneral stage. However, in the present studies it was more convenient to offer the infected blood meal for a limited time after the fly emergence.

Development of chancres or local skin reactions to T. congolense was shown to occur in rabbits but not guinea pigs or rats. This is in agreement with earlier studies using T. brucei organisms (Willett and Gordon, 1957). Mice bitten by T. brucei infected G. m. morsitans have also been shown to fail to develop chancres (Emery and Moloo, 1981). At post-mortem of the mice it was shown that the blood pool in which the fly fed, was located in the abdominal muscle and not in the subcutis (Emery and Moloo, 1981).

In rabbits, chancres developed at sites bitten by infected flies but not at sites bitten by non-infected flies. Local skin reactions of greater than 5 mm were obtained in over 85% of feeds. This compares with 86-91% chancre-producing rate reported by others in cattle and rabbits (Gray and Luckins, 1980; Akol and Murray, 1982). Non-infected flies usually produced local reactions <5 mm in diameter. In the few cases (11%) where the local skin reactions to uninfected tsetse bites exceeded 5 mm in diameter, the maximum diameter reached was <10 mm and duration of reaction was 3-5 days compared to 7-12 days for reactions at infective tsetse bites. The local skin reactions at sites bitten by uninfected flies might be due to bacteria deposited by the probing fly since older flies were observed to contain many bacteria in their proboscides.

Measurement of the diameter of hyperaemia and induration at the site of infective tsetse bites was found to be a reliable method of monitoring development of chancres. This method probably monitors both the cellular and pharmacological aspects of the chancre, unlike measurements of chancre skin thickness (Emery and Moloo, 1981; Akol and Murray, 1982), which probably monitors the cellular aspects only.

Experiments on the course of cyclical infection with T. congolense TREU 1690 in the rabbit have shown that up to seven weeks after infection there were no serious pathogenic effects. The lowest PCV of infected group was 30%. A similar minimum PCV has been reported in rabbits with T. congolense infections initiated by bloodstream forms (Holmes, 1976).

## CHAPTER FIVE

EFFICACY OF CHEMOTHERAPY AND CHEMOPROPHYLAXIS  
IN ANIMALS INFECTED WITH CYCLICALLY TRANSMITTED  
TRYPANOSOMA CONGOLENSE5.1 Introduction

Chemotherapy forms an important component of control methods used against African animal trypanosomiasis and a number of compounds are available including curative drugs such as diminazene aceturate, homidium, quinapyramine sulphate and suramin, and prophylactic drugs such as quinapyramine sulphate and chloride (Antrycide - prosalt), pyrrithidium bromide and isometamidium chloride. Unfortunately, to a varying extent, all trypanocidal drugs are affected by the development of stocks of trypanosomes which are resistant to doses of drugs at normal therapeutic levels. This is likely to become an increasingly serious problem as no new trypanocidal drugs have been developed since 1960 (Anon, 1979).

New trypanocidal drugs are difficult to discover and costly to develop for field use. Estimates show that 10,000 compounds have to be screened to obtain one or two which are promising but which have only a 10% chance of successful development (Anon, 1979). The reason is that most of the search for new trypanocidal compounds is empirical since little is known about the metabolism of trypanosomes. Even the modes of action of the currently used drugs are not known with certainty (Williamson, 1970).

Great care must therefore be exercised in the use of presently available drugs to minimise development of drug resistance. In many affected countries, continual surveillance for drug resistance is



mounted and appropriate changes made in the drug regimen when problems are discovered. Due to resistance problems with other trypanocidal drugs, diminazene aceturate and isometamidium chloride are the most commonly used trypanocidal drugs (Finelle, 1973; Holmes and Scott, 1982).

#### 5.1.1 Diminazene aceturate (Berenil)

The trypanocidal activity of diminazene aceturate, B.Vet.C. (Berenil, Farbwerke Hoechst AG, Frankfurt-Main) was first described nearly 30 years ago (Bauer, 1955; Fussgänger, 1955). The drug is available commercially as a yellow odourless powder soluble 1/14 in water at 20°C (Williamson, 1970). Prepared solutions are stable for up to 15 days because of an added antipyrine (Fairclough, 1963a). The recommended dosage for cattle infected with T. congolense is 3.5 mg/kg, given intramuscularly as 7% solution.

Diminazene aceturate is the most effective of the trypanocidal drugs (Williamson, 1970). This is due to its high therapeutic index, especially in cattle, its activity against trypanosomes resistant to other trypanocidal drugs and in spite of widespread usage the frequency of development of trypanosomes resistant to its action is very low.

(a) Mode of action of diminazene aceturate: There are conflicting reports on the mode of action of diminazene aceturate. Several studies indicated that the drug has in vitro trypanocidal activity against T. congolense and T. brucei (Bauer, 1958; Hawking, 1958; Goodwin and Tierney, 1977). In contrast, Maxie and Losos (1977) reported the absence of in vitro trypanocidal activity against T. congolense in blood obtained from infected animals up to 30 minutes after treatment with diminazene aceturate.

Results from some studies suggest that diminazene aceturate acts by presenting trypanosomes to the host immunological defence system. In cattle, significant rises in parasitaemia 5-10 minutes after diminazene aceturate injection suggested that parasites localised in the microcirculation are released into the general circulation, thus making them available to the host defence mechanism (Maxie and Losos, 1977). This has been confirmed in studies in which diminazene aceturate was compared with other trypanocidal and non-trypanocidal drugs for the ability to disperse trypanosomes located in blood capillaries in cattle and sheep (Mills et al., 1980; Kalu, 1983; Kalu, Ayandele and Aina, 1983). It was observed that those trypanocidal drugs which increased parasite concentrations in the jugular vein were the ones which were normally effective against T. congolense (Kalu et al., 1983).

The rapidity with which trypanosomes are killed in vivo by diminazene aceturate has been the subject of several studies. Bauer (1958) failed to isolate trypanosomes from infected cattle and dogs five hours after intramuscular injection of 3 mg/kg diminazene aceturate. Fairclough (1963b) isolated viable trypanosomes from a T. congolense-infected bovine at six hours but not at 12 hours after subcutaneous injection of 3.5 mg/kg diminazene aceturate even though parasites could still be detected in the treated animal 24 hours after treatment.

(b) Relapses of infections after treatment with diminazene aceturate: When an animal is treated with a curative dose of a trypanocidal drug and a relapse occurs in circumstances precluding re-infection, the development of drug resistance is to be suspected. It is possible however that parasites residing in tissue sites may be exposed to subcurative levels of the drug and these organisms might be

responsible for the relapses. One such form of chemotherapeutic failure was reported to occur in T. brucei-infected mice treated with diminazene aceturate at periods of more than a week after infection (Jennings, Whitelaw and Urquhart, 1977). Treatment three or seven days after infection resulted in complete cure while treatment 14 days or more after infection resulted in relapses 20-60 days later. In subsequent work it was shown that trypanosomes could be isolated from the brains of drug-treated mice but not from other organs or blood (Jennings et al., 1979). Mice treated with a combination of diminazene aceturate and nitroimidazole had a higher cure rate than mice similarly treated with diminazene aceturate alone (Jennings et al., 1980). Since some nitroimidazoles are known to cross the blood-brain barrier it was suggested that trypanosomes were located in the brain beyond the blood-brain barrier. The importance of this form of relapse in the field is unclear.

(c) Prophylactic effects: Early reports on the pharmacokinetics of diminazene aceturate indicated that the drug was rapidly excreted (Fussgänger and Bauer, 1958; 1960; Hawking, 1958). As a consequence, the drug was used in a number of field experiments in which this property was assumed (e.g. Whiteside, 1962). This assumption led to the belief that diminazene aceturate was unlikely to induce drug resistance on its own since subcurative dose levels would exist in the treated animal for only short periods (Fussgänger and Bauer, 1960; Williamson, 1970). Further studies have now indicated that the duration of detectable prophylactic effects of diminazene aceturate may depend on the species of trypanosomes as well as the route of inoculation used for challenge.

Hawking (1958) tested for residual trypanocidal activity by observing the motility of bloodstream T. rhodesiense incubated for 24 hours with plasma from a drug-treated rabbit. On this criterion, no trypanocidal activity could be detected in plasma collected 24 hours after treatment with 15 mg/kg diminazene aceturate. However, utilising the same in vitro technique using T. brucei and incubating the trypanosomes for up to 48 hours, Goodwin and Tierney (1977) found detectable trypanocidal activity in rabbit plasma for two weeks and in tissue fluid for up to one week after intramuscular injection of 40 mg/kg diminazene aceturate. More recently, rabbits injected intramuscularly with radiolabelled diminazene aceturate at 3.5 mg/kg dose rate, were shown to retain the drug in both plasma and tissue fluid for up to seven days after treatment (Gilbert and Newton, 1982; Gilbert, 1983).

In cattle, a chemical assay technique showed that after intramuscular injection of 2.5 mg/kg diminazene aceturate, the drug could not be detected in plasma taken 24 hours after treatment (Klatt and Hajdu, 1976). Biological assay techniques utilising T. brucei have however revealed that diminazene aceturate is retained in cattle for long periods after treatment. By using serum from treated cattle as diluent for carrying out infectivity titrations with a stock of T. brucei it was shown that animals treated with 7 mg/kg diminazene aceturate retained levels of the drug which had trypanocidal activity for up to 21 days after treatment (van Hove, Cunningham and Grange, 1965a,b).

In other studies on the effect of diminazene aceturate, treated animals were challenged with trypanosomes and the development of

parasitaemia monitored. Results depended on the species of trypanosomes used in the challenge. In all studies in which T. brucei spp. were used, diminazene aceturate was reported to have prolonged prophylactic effect. Mice treated with 91 and 45 mg/kg diminazene aceturate were shown to be resistant to challenge with T. brucei for 42 and 33 days after treatment respectively (Lumsden, Herbert and Hardy, 1965). In rats, treatment with 50 mg/kg diminazene aceturate conferred prophylactic effect of 49 days against T. rhodesiense (Raether et al., 1972) while at 5 mg/kg dosage rate the prophylactic effect lasted for 28 days against T. brucei (Zahalsky and Weinberg, 1976). Also, monkeys treated with 5-20 mg/kg diminazene aceturate were shown to be resistant to challenge with T. rhodesiense 7-21 days after treatment (Raether, Hajdu and Damm, 1974).

In contrast, diminazene aceturate appears to have little prophylactic effect against T. congolense. Mice were shown to be only partially protected when inoculated with T. congolense five hours after treatment with 10 mg/kg diminazene aceturate (Fink and Damm, 1974). Mice treated with 50 mg/kg diminazene aceturate were still susceptible to cyclically transmitted T. congolense one week after treatment (Nantulya, Doyle and Jenni, 1980b). At 40 mg/kg diminazene aceturate did not completely protect mice against challenge with bloodstream T. congolense three days after treatment (Whitelaw, 1982).

It thus seems that in small laboratory animals, the prophylactic effect of diminazene aceturate against T. congolense is of much shorter duration than that against T. brucei. James (1976) showed that the minimum concentration of diminazene aceturate required to abolish infectivity of T. congolense after 12 minutes exposure at 37°C

is twice that for T. brucei. This indicates that there may be differences between the actions of diminazene aceturate on these two trypanosome species. The result was however complicated by the use of two different host species, rats and mice, for testing the infectivity of T. brucei and T. congolense respectively.

In cattle, it was found possible to infect animals with T. congolense bloodstream forms 24 hours after treatment with 3.5 mg/kg diminazene aceturate. However, when the dose was doubled, a prophylactic effect of at least 24 hours could be detected (Fairclough, 1963b). These observations have recently been extended by Wellde and Chumo (1983) who found that at 7 mg/kg, diminazene aceturate protected cattle against intravenous inoculation of  $10^5$  bloodstream forms of T. congolense for up to 12 days after treatment.

#### 5.1.2 Isometamidium chloride

Isometamidium chloride, B.vet.C. (Samorin, May and Baker Ltd., England) has been used as a prophylactic trypanocidal drug since 1961 (Williamson, 1970). It is available as a dark brown powder which readily dissolves in cold water. For curative purposes dose rates of 0.25-0.5 mg/kg suffice in cattle. Doses of 0.5-1.0 mg/kg are claimed by the manufacturer to give a prophylactic effect of 3-6 months in cattle, depending on the degree of trypanosome challenge.

There are few studies on the pharmacokinetics of isometamidium. It has been reported that it is less active in vitro than in vivo against T. congolense and T. rhodesiense (Hill, 1965). The importance of depots of isometamidium chloride at the injection site in conferring a prophylactic effect against T. congolense has been studied in rats

and mice (Hill and McFadzean, 1963). The drug was injected subcutaneously into the tails of rats and within 24 hours of treatment the tails of some animals were amputated. Rats with amputated tails were protected against infection with  $2 \times 10^5$  T. congolense for only one week after treatment while those with tails were protected for 16 weeks.

Further studies in rats have shown that after intravenous injection isometamidium was rapidly removed from the blood and was found in the liver and kidney (Philips et al., 1967). These studies in rats have been corroborated by studies in goats (Braide and Eghianruwa, 1976). Sera from animals treated with 0.5 mg/kg isometamidium intravenously did not contain detectable concentrations of drug 24 hours after treatment. On the other hand, serum from goats treated with the same dose intramuscularly contained detectable levels of isometamidium at 24 hours but not at two or more weeks after treatment. High levels of isometamidium were detectable in liver and kidneys for up to four weeks after intramuscular injection of 0.5 mg/kg isometamidium.

There have been several field trials on the prophylactic effect of isometamidium chloride. In one such trial in East Africa, Robson (1962) found that isometamidium methane sulphonate at 2.0 mg/kg dose rate gave a mean protection period of 258 days (range 93-398 days). Metamidium, which contains 45% isometamidium (Williamson, 1970), gave a mean protection of 226 days (range 23-398 days). Mean time of development of infections in control animals was 38.5 days as determined by thick blood smear examination.

In a similar trial in West Africa, Kirkby (1964) reported a protection period of 97-122 days after treatment with isometamidium chloride



at 1-2 mg/kg. Subsequent studies in East Africa have shown that the protection period in beef cattle after intramuscular injection of 1-2 mg/kg isometamidium may be as short as ten weeks (Wiesenhutter, Turner and Kristensen, 1968).

It is clear that few laboratory studies have been carried out on the efficacy of diminazene aceturate and isometamidium chloride against cyclically transmitted T. congolense. In natural infections it is now recognised that T. congolense undergoes development extra-vascularly at the sites of the formation of trypanosomal chancres (Roberts, Gray and Gray, 1969; Gray and Luckins, 1980; Akol and Murray, 1982). Since drugs normally attain lower levels in tissue, it is possible that diminazene aceturate might be less effective against infection in which trypanosomes induce chancre formation compared with infections in which chancres do not occur such as after intravenous inoculation. Similarly, the prophylactic effect of diminazene aceturate and isometamidium chloride might be reduced in cyclically transmitted infections where chancre formation occurs. The following experiments were designed to investigate the curative and prophylactic effects of diminazene aceturate and isometamidium chloride against cyclically transmitted T. congolense infections in rabbits.

## 5.2 Gross and Microscopic Effects of Trypanocidal Drug Treatment on Trypanosoma congolense Chancres

Experimental design: Chancre development was followed in three rabbits 826, 831 and 821 bitten by 1-3 G. m. morsitans infected with T. congolense TREU 1457. Rabbits 826 and 831 were killed 14 and 21 days respectively after infection to provide samples for histological



examination. Rabbit 821 was observed for at least 42 days after infection.

Two rabbits, 803 and 809 were treated with 7 mg/kg diminazene aceturate at two and five days respectively after bites from 12 and 11 G. m. morsitans infected with T. congolense TREU 1457. Chancre development at the bite sites was monitored for 21 days after the infective tsetse bites.

Four rabbits, 823, 855, 817 and 825 were infected with T. congolense TREU 1457 by bites from 4-5 infected G. m. morsitans. Rabbits 823 and 855 were treated with 7 mg/kg diminazene aceturate and 5 mg/kg homidium bromide respectively at day 6 post-infection. Rabbits 817 and 825 were treated with 7 mg/kg diminazene aceturate at day 8 post-infection. Chancre development was monitored in the four rabbits up to ten days post-infection when they were killed to provide samples for histology.

Specimens for histological studies were obtained from another nine infected and treated rabbits and 12 infected untreated rabbits in addition to those rabbits mentioned above. The times of treatment and days on which the rabbits were killed for the preparation of histological sections are shown in Tables 5.1A and 5.1B. Sections of skin from rabbit 1208 killed on the same day on which treatment was carried out, were obtained three hours after treatment. Removal of chancres and fixation in Bouin's fixative was carried out immediately after death by cervical dislocation. The skin samples were processed, embedded in paraffin wax and 5  $\mu$ m sections were cut and stained by the Giemsa-colophonium technique as detailed in Appendix 2.

Results: Diameters of chancres at different times after infection in untreated control animals and in animals treated with trypanocidal drugs at different days after infection are shown in Figures 5.1 and 5.2. In untreated animals, chancres were first seen five days after infection and reached maximum size 5-7 days later. A second smaller increase in size was recorded in two rabbits, 826 and 831, 14 and 15 days after infection (Figures 5.1A and 5.1B). In all rabbits chancres disappeared 16-18 days after infection.

Trypanocidal drug treatment prevented the development of the trypanosomal chancre. In rabbit 803, treated on day 2 after infection, no chancres developed at any of the sites where 12 infected flies fed (Figure 5.2A). In rabbit 809 treated on day 5 after bites from 11 infected flies, small chancres with mean maximum diameters of 8 mm, 10 and 11 days post-infection were recorded (Figure 5.2B). In rabbits 823 and 855 treated at seven days post-infection, the further development of the chancre was prevented although a slight increase in diameter was seen on the following day (Figures 5.2C and 5.2D).

Treatment on day 8 after infection produced different results in the two rabbits examined. In one rabbit (817) the mean chancre diameter progressively decreased in the following two days while in the other (825) there was progressive increase during the same period (Figures 5.2E and 5.2F).

In the histological studies, in sections removed from untreated rabbits, 5-14 days after infection, trypanosomes were readily detectable within and between collagen bundles which appeared disrupted. Trypanosomes were particularly numerous in sections obtained at 7-10 days after infection (Plates 5.2A and 5.2B). Trypanosomes could not

be detected in sections removed from rabbits on days 4 and 21 after infection (Plates 5.1A and 5.1B). Plate 5.2A also shows that there was an extensive cellular reaction on day 8, but that it was declining after day 10 although it was still visible by day 21 (Plate 5.1B) when the chancre was not discernible macroscopically.

Trypanosomes were not seen in skin removed 2-4 days after treatment which had been carried out on days 3-9 after infection. However, the cellular reaction was comparable in extent and composition, to that in chancres removed at a similar period post-infection, from untreated rabbits. Mononuclear cells were the predominant cell type seen.

Sections removed from rabbit 1207 24 hours after treatment on day 9 post-infection revealed degenerating trypanosomes (Plate 5.3B). A smear made from chancre fluid obtained from this rabbit three hours after treatment, had shown numerous trypanosomes (Plate 5.3A). Chancres removed from another rabbit (1208), three hours after treatment on day 9 post-infection revealed the presence of numerous intact trypanosomes in histological sections (Plates 5.4A and 5.4B).

There was no evidence of readily detectable phagocytosis in sections from treated animals.

Table 5.1

Trypanosome-infected rabbits from which skin sections were obtained for histological examination of the effects of drug treatment on chancre development.

A. Animals treated with 7 mg/kg DIMINAZENE ACETURATE or 5 mg/kg HOMIDIUM BROMIDE

Rabbit Number	Infected and Treated		Infected and Untreated	
	Day treated	Day killed	Rabbit Number	Day killed
824	3	7	857	4
822	5	7	801	5
823	7	10	829	8
855*	7	10	879	9
817	8	10	830	10
825	8	10	826	14
1066	10	12	831	21

\*Treated with Homidium bromide

B. Animals treated with 14 mg/kg DIMINAZENE ACETURATE

Rabbit Number	Infected and Treated		Infected and Untreated	
	Day treated	Day killed	Rabbit Number	Day killed
1052	3	5	1051	5
1053	5	7	1054	7
1060	8	10	1062	9
1207	8	9	1065	10
1208	9	9	1064	11
1055	9	11		

Figure 5.1 Mean diameters of chancres on three untreated rabbits cyclically infected with T. congolense TREU 1457.

Key

A = Rabbit 826

B = Rabbit 831

C = Rabbit 821

K = Killed

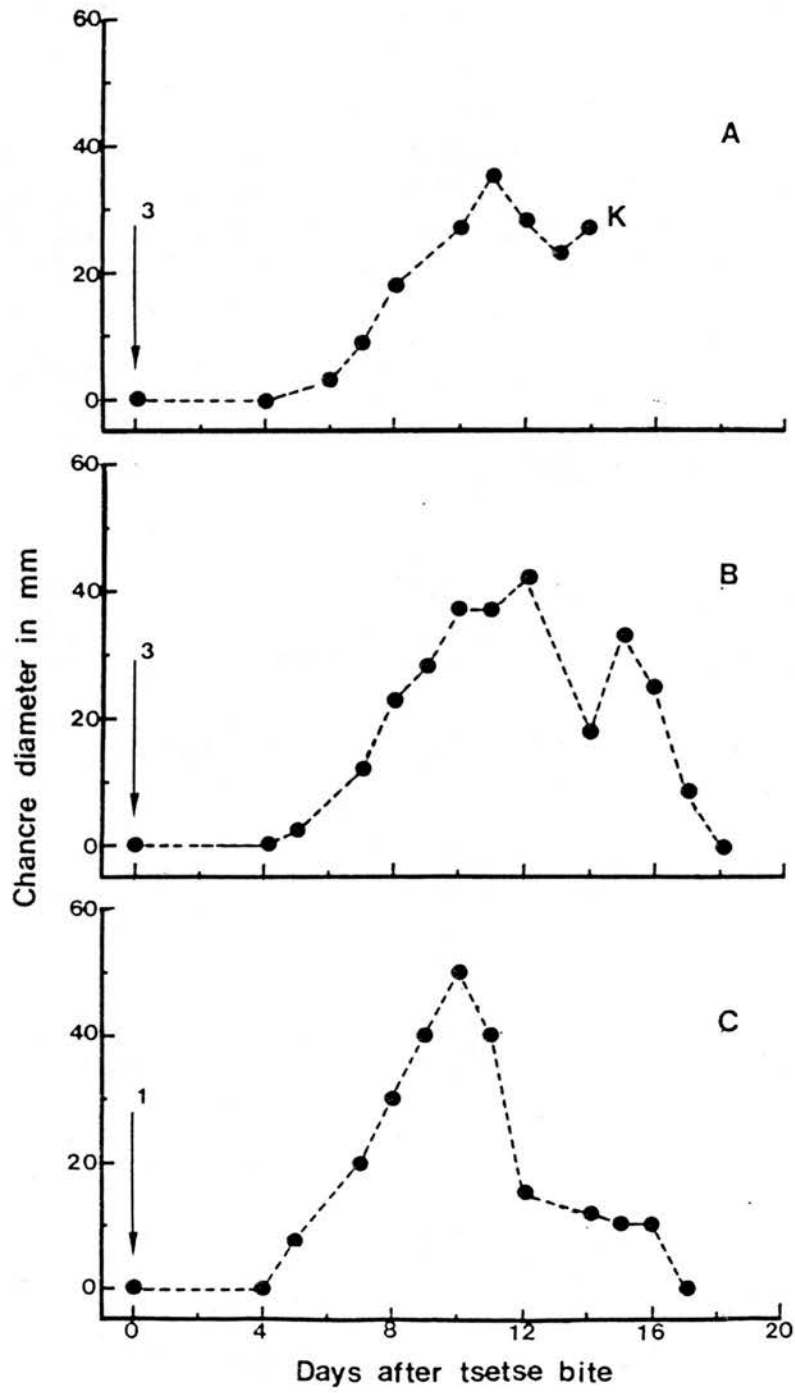
FIG. 5.1

Figure 5.2 Mean diameters of chancres on rabbits cyclically infected with T. congolense TREU 1457 and then treated with diminazene aceturate or homidium bromide.

Key

A = Rabbit 803

B = Rabbit 809

C = Rabbit 823

D = Rabbit 855

E = Rabbit 817

F = Rabbit 825

K = Killed

↓ = Infective tsetse bites (Number of flies fed is indicated by the number above the arrow)

**B**  
▼ = Treatment with diminazene aceturate

▼ = Treatment with 5 mg/kg homidium bromide

FIG. 5.2

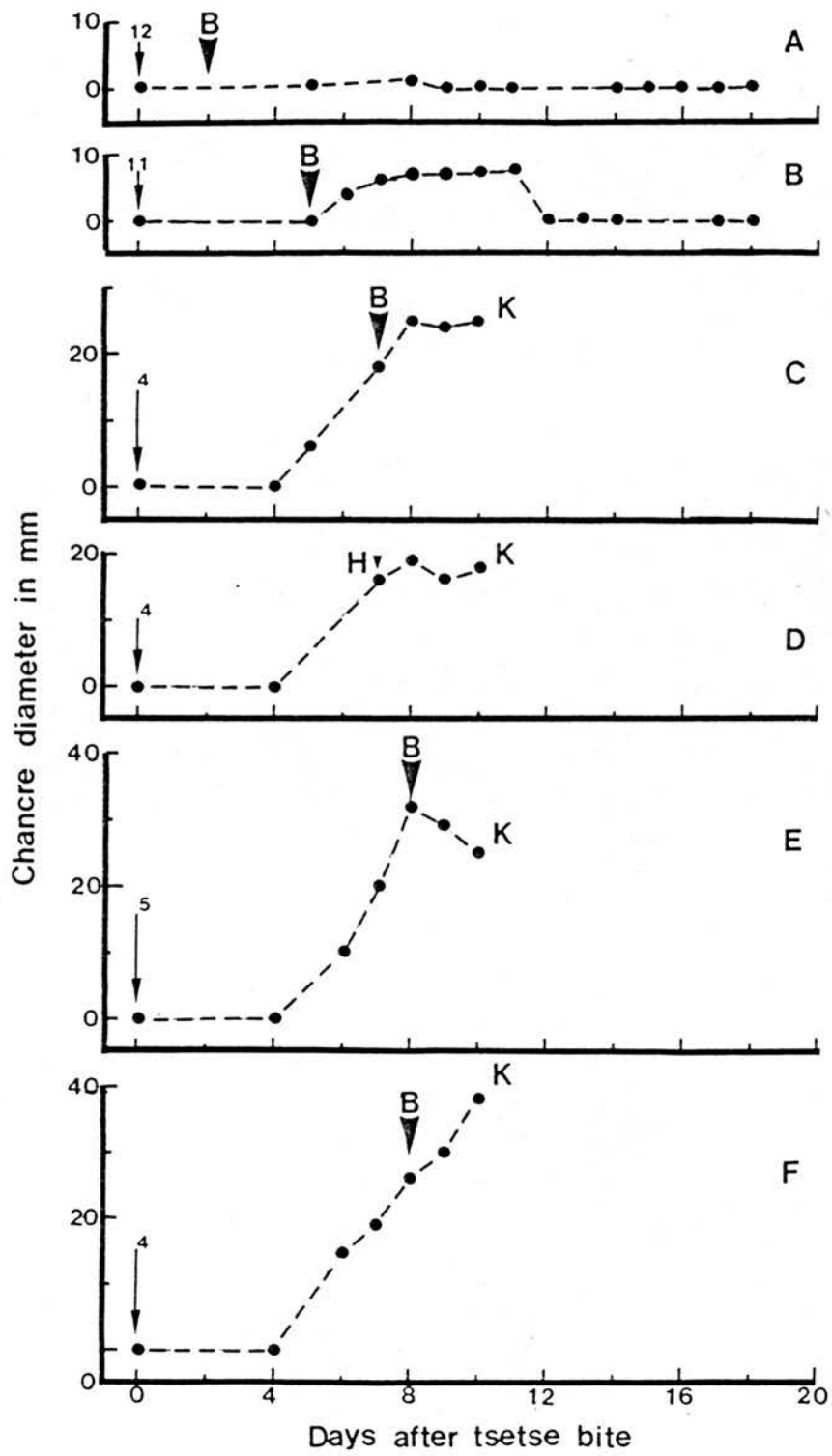




Plate 5.1A Section through skin removed from site of infective bite on Rabbit 857 at day 4 post-infection, showing scanty cellular reaction.  
(Giemsa - colophonium x 312)

Plate 5.1B Section through area of local skin reaction on Rabbit 831, 21 days post-tsetse bite.  
(Giemsa - colophonium x 312)

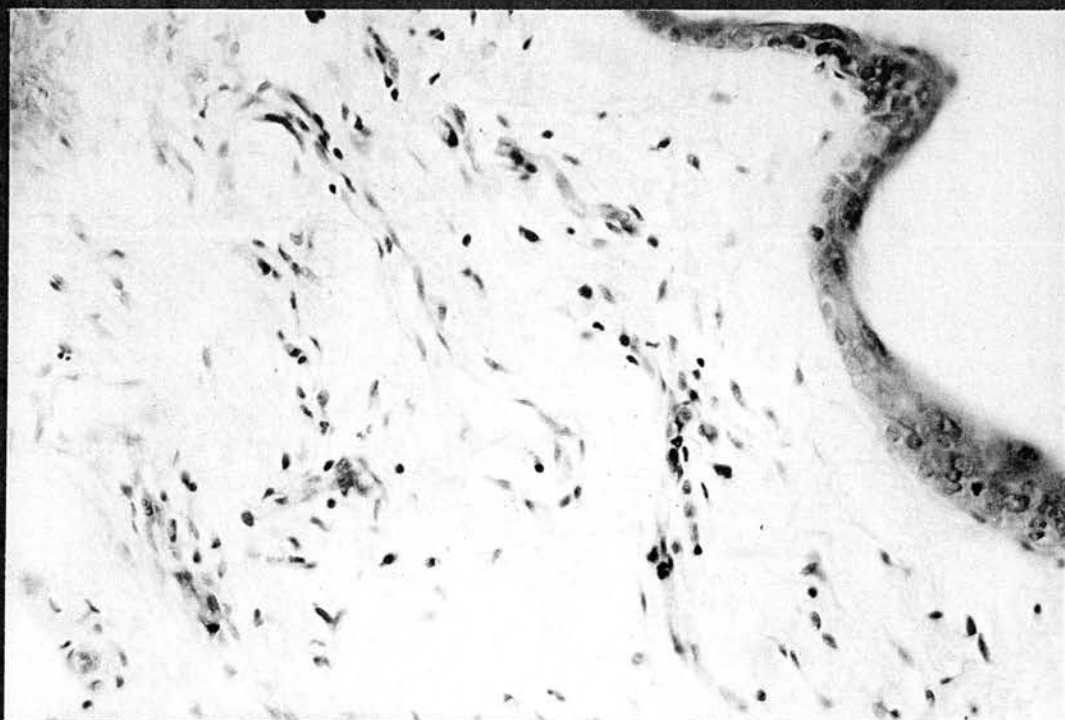
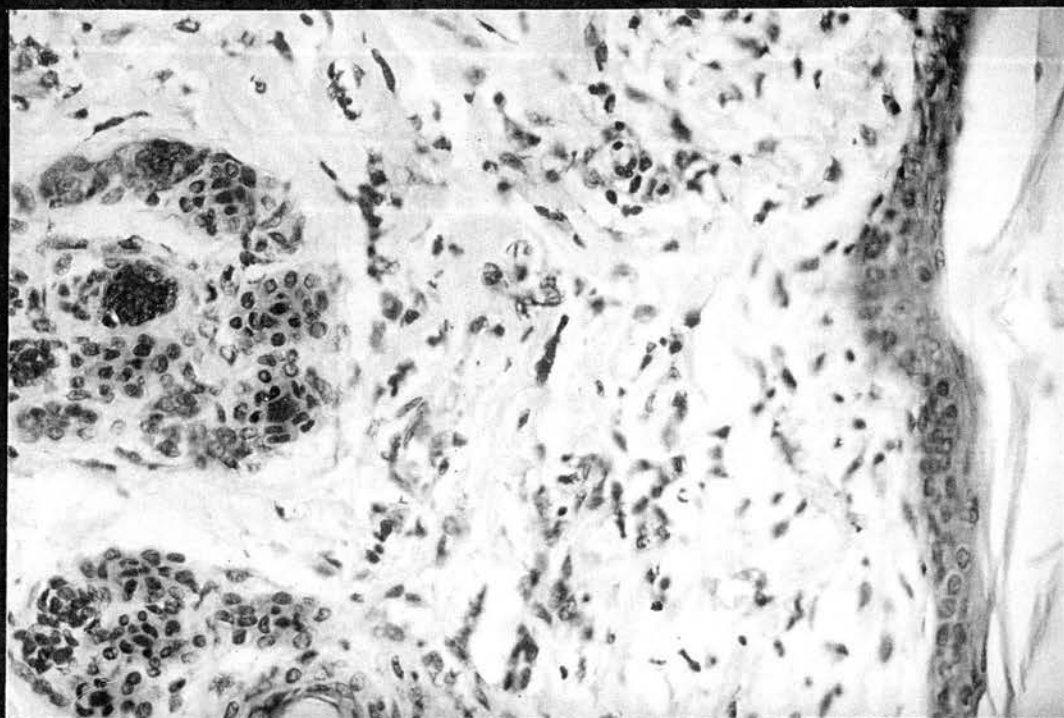


Plate 5.2A Section through a local skin reaction removed from rabbit 829 at day 8 post-tsetse bite, showing massive cellular reaction particularly in papillary dermis.  
(Giemsa - colophonium x 125)

Plate 5.2B Higher magnification of Figure 5.2A showing trypanosomes (arrowed) in collagen fibres.  
(Giemsa - colophonium x 625)

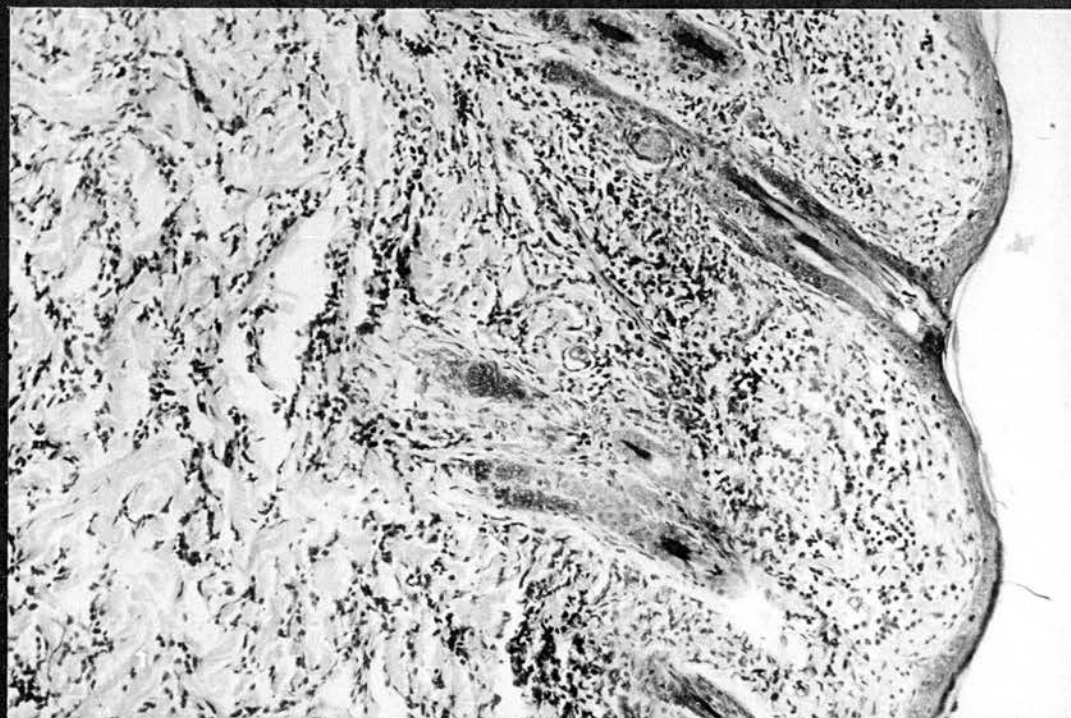
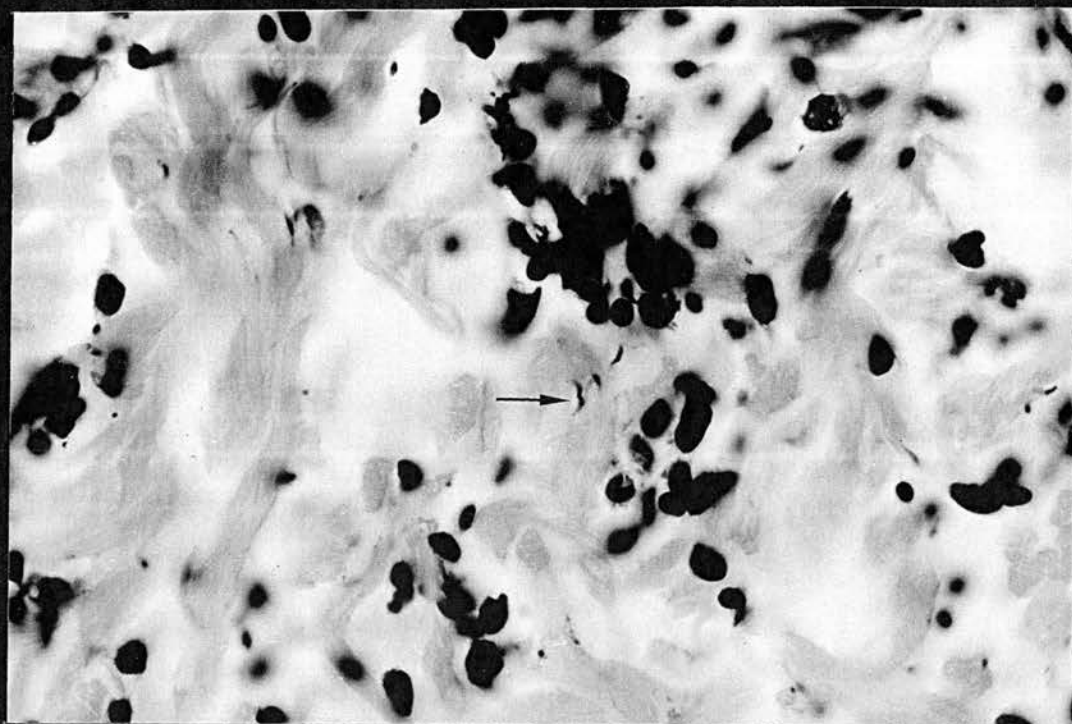
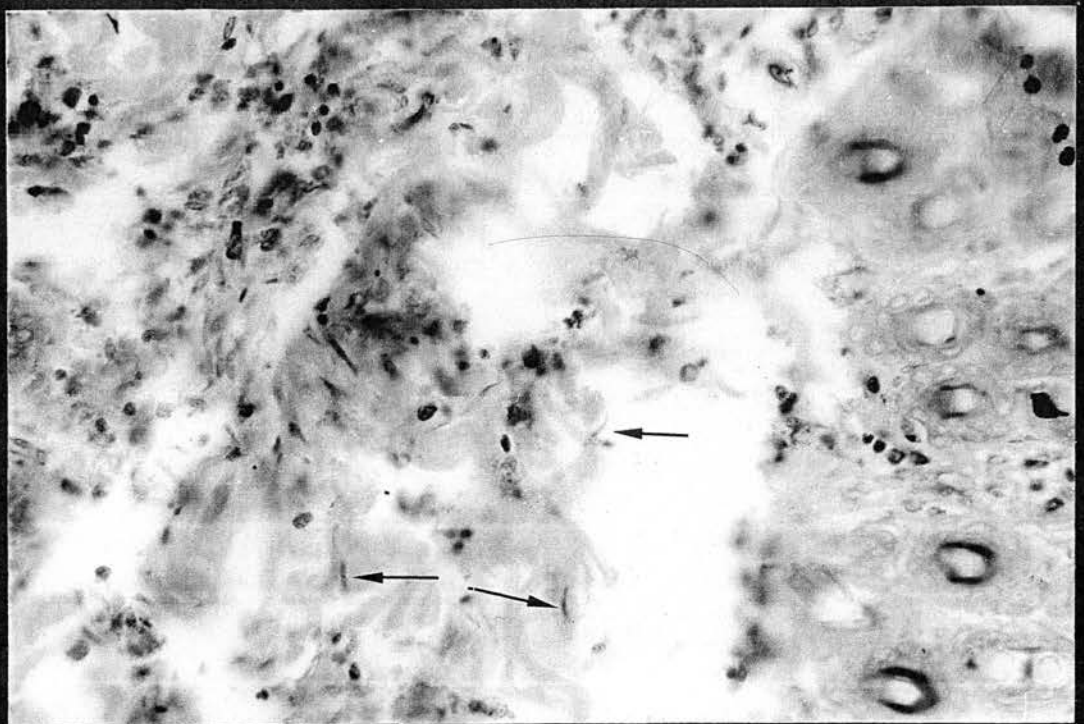


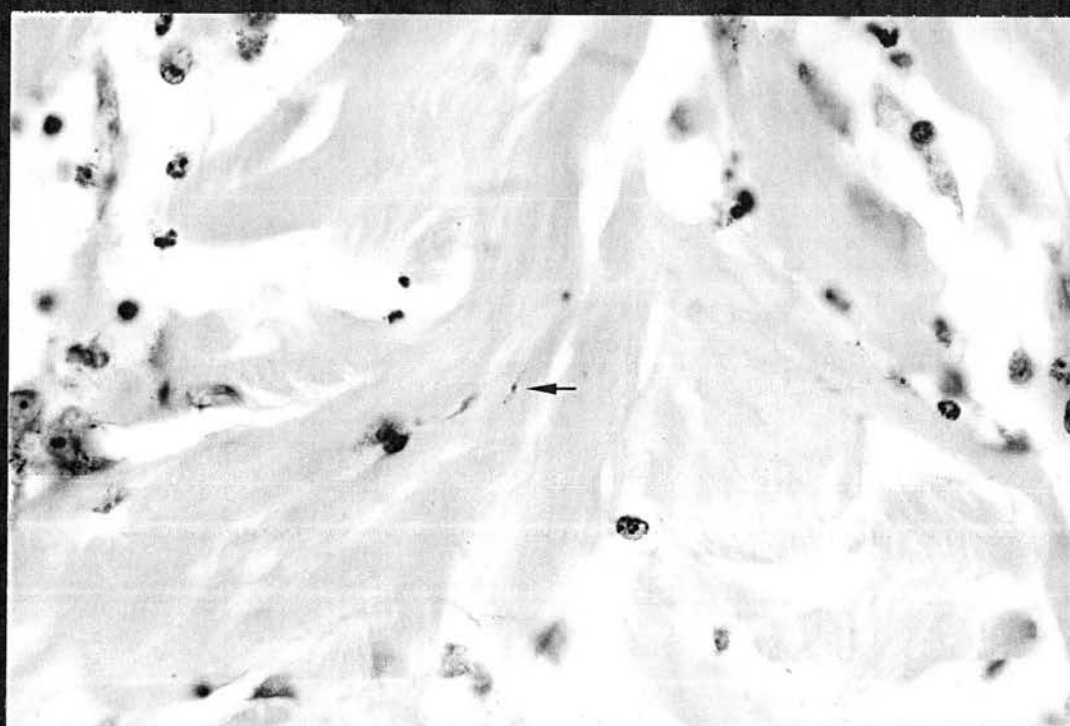
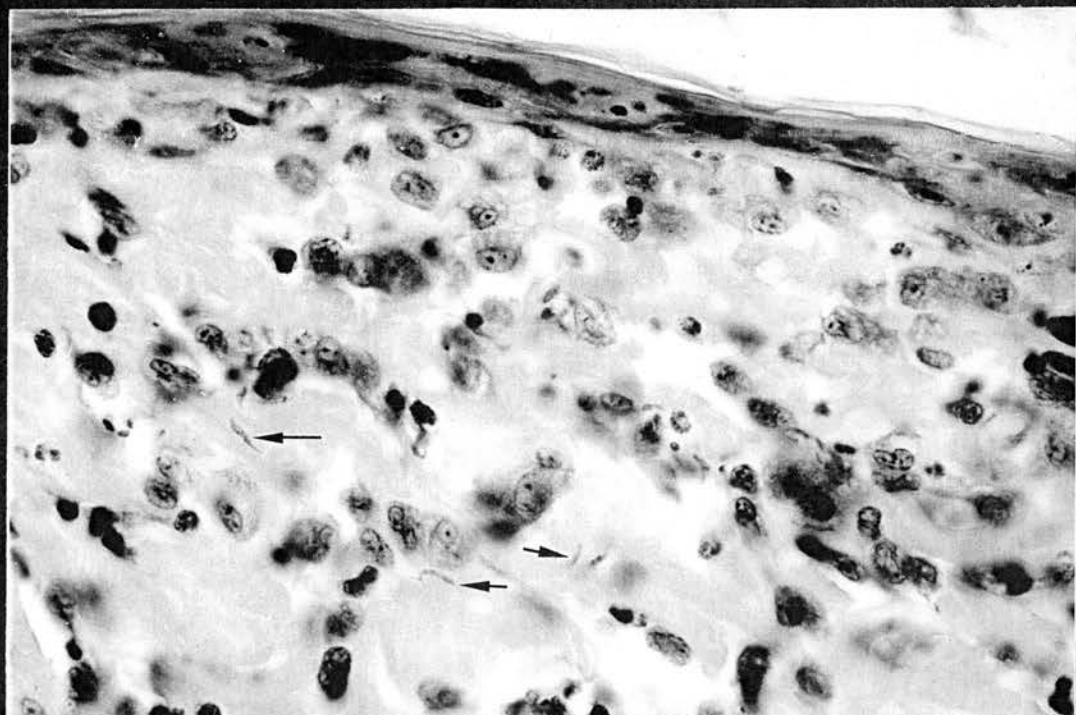
Plate 5.3A Trypanosomes in smear of chancre fluid removed from rabbit 1207, three hours after treatment with diminazene aceturate.  
(Giemsa, x 625)

Plate 5.3B Section through chancre removed from rabbit 1207, 24 hours after treatment with diminazene aceturate showing degenerating trypanosomes (arrowed).  
(Giemsa - colophonium x 312)





Plates 5.4A and B Sections through chancre removed from  
rabbit 1208 three hours after treatment  
with diminazene aceturate, showing  
trypanosomes (arrowed) in collagen fibres.  
(Giemsa - colophonium x 625)





### 5.3 Assessment of Efficacy of Diminazene Aceturate Treatment

#### Experiment 1

Experimental design: Six rabbits were treated with 7 mg/kg diminazene aceturate 3-8 days after cyclical infection with T. congolense TREU 1457 or TREU 1690. For comparative purposes, one rabbit cyclically infected with T. congolense TREU 1457 was treated with 5 mg/kg homidium bromide on day 7 after infection. To assess the efficacy of treatment, attempts were made to isolate trypanosomes from chancres and blood after treatment. Two to four days after treatment, blood was inoculated in 0.5 ml amounts into each of three mice. The hair over the site of skin reaction area was then closely shaved with hair clippers and a razor blade, and the rabbit then killed by cervical dislocation. The chancre was immediately removed and kept moist with ice-cold PBSG (pH 8.0). A suspension was prepared by scraping and teasing all layers of the chancre into 3.0 ml of cold PBSG. The suspension was then lightly centrifuged (100 g) for 20 minutes and the supernatant injected intraperitoneally into each of five mice in 0.5 ml amounts. Mice were subsequently checked for the presence of trypanosomes by the wet smear method for up to 35 days after inoculation. Five infected, untreated control rabbits were killed at 4-7 days after cyclical infection and attempts made to isolate trypanosomes from chancres as described above.

Results: Inoculation of material from the site of tsetse bite and from blood showed that in untreated rabbits it was possible to isolate trypanosomes from the chancre as early as four days after infection and from the blood as early as five days after infection (Table 5.2). However, no trypanosomes could be isolated from chancres or blood from any of the treated rabbits.

## Experiment 2

Experimental design: Two groups of rabbits, Group I and II comprising 17 and 16 animals respectively were monitored for development of relapse infections after treatment of cyclically transmitted T. congolense infections with 7 mg/kg diminazene aceturate. In Group I, nine rabbits infected with T. congolense TREU 1690 and seven rabbits infected with T. congolense TREU 1682 were treated between day 0 and day 42 after infection. One rabbit, 885 was infected with both T. congolense TREU 1682 and TREU 1690 and treated 42 days after infection.

Rabbits in Group II were infected with T. congolense TREU 1690 and then treated with 7 mg/kg diminazene aceturate 7-28 days after infection.

Prior to treatment, the number of chancres on each animal was noted and the presence of trypanosomes in blood checked by microhaematocrit/buffy coat technique (Murray et al., 1977). After treatment, blood was checked twice weekly by the microhaematocrit/buffy coat technique from 14-42 days after which the absence of infection was confirmed by subinoculation of 0.5 ml of blood into each of three mice.

Results: In Group I, rabbits 878 and 883 infected with T. congolense TREU 1690 and rabbit 879 infected with T. congolense TREU 1682 treated at eight or nine days post-infection showed relapses of parasitaemia after treatment (Table 5.3). In Group II, two animals, 960 and 984 treated at day 10 post-infection developed relapses after treatment (Table 5.4). All other animals were cured, irrespective of the time of treatment.

### Experiment 3

Experimental design: Three pairs of rabbits infected with T. congolense TREU 1690 were treated with 14 mg/kg diminazene aceturate eight, nine or ten days after infection. Five other pairs of rabbits infected with T. congolense TREU 1690 were treated with 7 mg/kg diminazene aceturate at seven, eight, nine, ten or 14 days after infection. Before treatment animals were examined for chancres and the blood checked for the presence of trypanosomes. After treatment animals were checked for parasitaemia twice a week by the microhaematocrit/buffy coat technique and the absence of trypanosomes was confirmed by the inoculation of blood in 0.5 ml amounts into each of three mice.

Results: Table 5.5 shows that two rabbits 1027 and 1037 treated with 7 mg/kg diminazene aceturate nine and ten days after infection respectively, had relapse infections after treatment. One rabbit, 1020 had relapse infection after treatment with 14 mg/kg diminazene aceturate at day 9 post-infection.

In the experiments described above, seven out of 24 rabbits treated with 7 mg/kg diminazene aceturate 5-16 days after infection had relapse infections after treatment. No relapse infections occurred in 19 rabbits treated with the same dose of diminazene aceturate 0-4 days or 17-42 days after infection (Table 5.6A).

Among the six rabbits treated with 14 mg/kg diminazene aceturate eight, nine or ten days after infection, one rabbit out of two treated at day 9 post-infection showed relapse parasitaemia after treatment (Table 5.6B).

Table 5.2

Development of infections in mice inoculated with homogenates of chancres or whole blood from untreated rabbits infected with Trypanosoma congolense TREU 1457 and rabbits infected with T. congolense TREU 1457 and treated with 7 mg/kg DIMINAZENE ACETURATE at different times after infection.

Rabbit Number	Day of Treatment Post-infection	Day of Tissue Removal Post-infection	Presence of Trypanosomes in Mice*	
			Chancres homogenate	Whole blood
857	-	4	+	0
801	-	5	+	+
1051	-	5	+	+
1054	-	7	+	+
1062	-	7	+	+
824	3	7	0	0
822	5	7	0	0
823	7	10	0	0
825	8	10	0	0
817	8	10	0	0
1066	10	12	0	0

0 = No trypanosomes detected by wet smear examination

Table 5.3

Development of parasitaemia in rabbits cyclically infected with *Trypanosoma congolense* TREU 1682 or TREU 1690 and then treated with 7 mg/kg DIMINAZENE ACETURATE at different days after infection.

Rabbit Number	Day of Treatment Post-infection	Trypanosome stock (TREU)	Number of chancres present on day of treatment	Presence of Trypanosomes	
				Before* treatment	After** treatment
896	0	1682	0	NC	0
882	2	1690	0	NC	0
881	4	1690	0	NC	0
893	4	1682	0	NC	0
880	6	1690	2	0	0
892	8	1682	1	0	0
878	8	1690	1	0	0
879	9	1682	5	0	+
883	9	1690	7	0	+
877	10	1690	1	0	+
889	12	1682	2	0	0
876	21	1690	0	+	0
886	21	1682	0	+	0
884	27	1690	0	+	0
874	42	1690	0	+	0
887	42	1682	0	+	0
885	42	1682 < 1690	0	+	0

\*0 = No trypanosomes detected by microhaematocrit/buffy coat technique

\*\*0 = No trypanosomes detected by mouse subinoculation

NC = Not checked

Table 5.4

Development of parasitaemia in rabbits cyclically infected with Trypanosoma congolense TREU 1690 and then treated with 7 mg/kg DIMINAZENE ACETURATE at different days after infection.

Rabbit Number	Day of Treatment Post-infection	Number of chancres present on day of treatment	Presence of Trypanosomes	
			Before* treatment	After** treatment
937	7	18	0	0
975	7	4	0	0
971	8	18	0	0
960	10	7	0	+
984	10	10	0	+
959	14	8	+	0
970	14	8	+	0
958	17	0	+	0
962	18	0	+	0
961	20	0	+	0
939	28	0	+	0
942	28	0	+	0
943	28	0	+	0
944	28	0	+	0
945	28	0	+	0
947	28	0	+	0

\*0 = No trypanosomes detected by the microhaematocrit/buffy coat technique

\*\*0 = No trypanosomes detected by mouse subinoculation

Table 5.5

Development of parasitaemia in rabbits cyclically infected with *Trypanosoma congolense* TREU 1690 and then treated with 7 mg/kg or 14 mg/kg DIMINAZENE ACETURATE at different days after infection.

Rabbit Number	Day of Treatment Post-infection	Number of chancres present on day of treatment	Drug dose (mg/kg)	Presence of Trypanosomes	
				Before* treatment	After** treatment
1058	7	4	7	0	0
1061	7	4	7	0	0
1056	8	4	7	0	0
1059	8	4	7	0	0
1057	8	4	14	0	0
1063	8	4	14	0	0
1027	9	5	7	0	+
1049	9	4	7	0	0
1020	9	4	14	0	+
1050	9	4	14	0	0
1037	10	6	7	0	+
1045	10	10	7	0	0
1047	10	5	14	0	0
1046	10	9	14	0	0
1044	14	3	7	+	0
1048	14	6	7	+	0

\*0 = No trypanosomes detected by microhaematocrit/buffy coat technique

\*\*0 = No trypanosomes detected by mouse subinoculation

Table 5.6

Summary of results on development of relapse parasitaemias in rabbits treated with DIMINAZENE ACETURATE at different times after cyclical infection with Trypanosoma congolense TREU 1682 or TREU 1690.

A. Treatment with 7 mg/kg DIMINAZENE ACETURATE

Day of Treatment Post-infection	Number of animals/ with chancres	Number of animals/ treated	Number of animals/Number of animals showing trypanosomes/ treated	
			At treatment	After treatment
0-4		0/4	0/4	0/4
5-16		24/24	5/24	*7/24
17-42		0/15	15/15	0/15

\*All seven treated 8-10 days after infection

B. Treatment at 14 mg/kg DIMINAZENE ACETURATE

Day of Treatment Post-infection	Number of animals/ with chancres	Number of animals/ treated	Number of animals/Number of animals showing trypanosomes/ treated	
			At treatment	After treatment
8		2/2	0/2	0/2
9		2/2	0/2	1/2
10		2/2	0/2	0/2



#### 5.4 Secondary Chancres Associated with Relapses in Parasitaemia after Chemotherapy

Normally, trypanosome chancres in untreated rabbits disappear between 14-19 days after infection. In experiments in determining the efficacy of diminazene aceturate, described above, it was observed that in some drug-treated rabbits in which relapses in parasitaemia occurred, secondary chancres sometimes developed at or near the sites of the primary chancres. It was therefore decided to extend the monitoring of chancres in drug-treated rabbits beyond 21 days after infection.

Experimental design: Sixteen rabbits treated with diminazene aceturate at 7 mg/kg or 14 mg/kg, 7-14 days after infection (Table 5.5) were monitored for the appearance of secondary chancre development for up to 35 days after infection. Presence of trypanosomes in blood before and after treatment was determined by microhaematocrit/buffy coat technique and subinoculation of blood into mice.

Results: One rabbit, 1027 showed relapse in parasitaemia after treatment but no evidence of secondary chancre development. Two rabbits, 1020 and 1037 treated at days 9 and 10 after infection, were each observed to develop one secondary chancre, followed by relapse parasitaemia post-treatment. The secondary chancre recurred near the centre of one of the sites of primary chancres. Figure 5.3 shows the mean diameters of the chancres on these two rabbits, 1020 and 1037, at different times after infection. It can be seen that in rabbit 1037 chancres had disappeared by day 15 after infection but another chancre was observed on day 23 after infection. In rabbit 1020 on the other hand, one chancre did not completely disappear and was observed

to increase in size again on day 21 after infection.

The rabbits which developed secondary chancres were killed at 29 and 33 days after infection to provide histological samples. Local skin reaction was removed, processed and tissue sections stained with Giemsa colophorium. Plates 5.5A, 5.5B, 5.6A and 5.6B show the cellular reaction in the secondary chancres from rabbits 1020 and 1037. When compared to the residual cellular reaction at day 21 post-infection in an untreated rabbit (Plate 5.1B), it can be seen that the reaction in a secondary chancre developing after treatment is very intense. The predominant cell type is the plasma cell (Plates 5.6A and 5.6B). However, an extensive search of histological sections failed to reveal the presence of trypanosomes.

Figure 5.3 Mean diameters of chancres on rabbits 1020 and 1037 at different days after bites by tsetse flies infected with T. congolense TREU 1690.

Key

▲ Rabbit 1020

● Rabbit 1037

**B**  
▼ or ▼ Treatment with diminazene aceturate

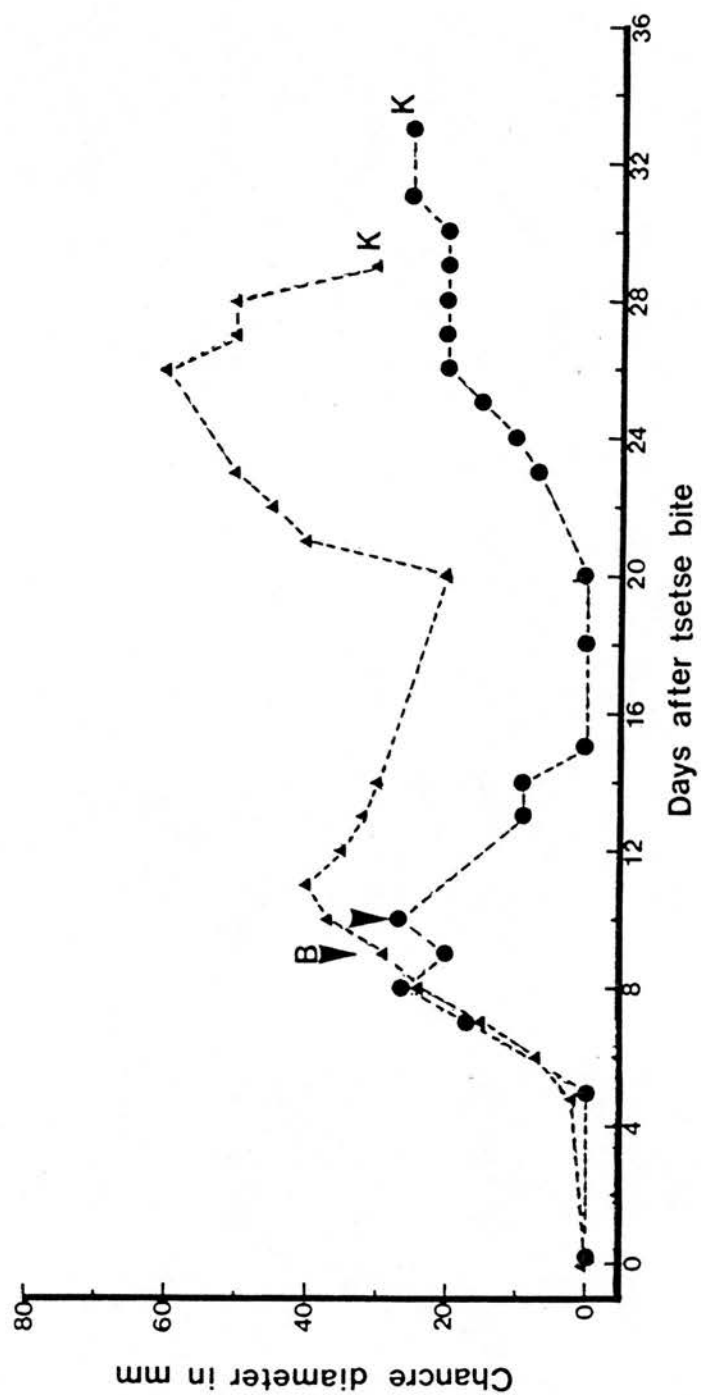
FIG. 5.3

Plate 5.5A Section showing massive cellular reaction in secondary chancre removed from rabbit 1020 at day 29 post-infection.  
(Giemsa - colophonium x 125)

Plate 5.5B Section through chancre removed from rabbit 1037, 33 days post-infection. Note massive cellular reaction particularly in papillary dermis.  
(Giemsa - colophonium x 125)

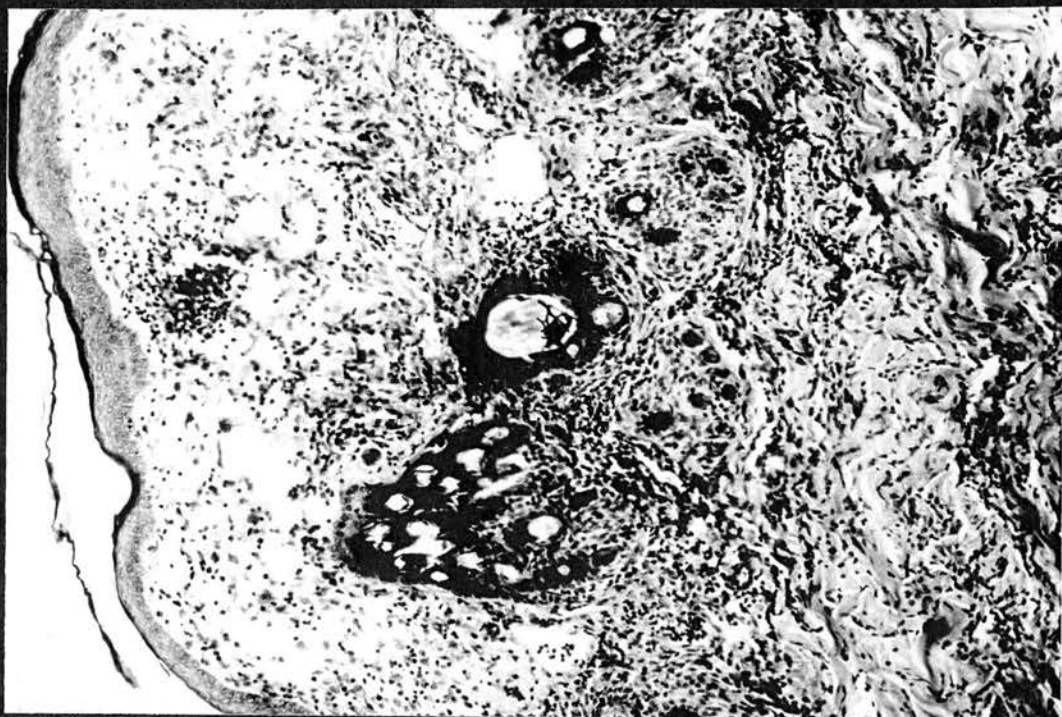
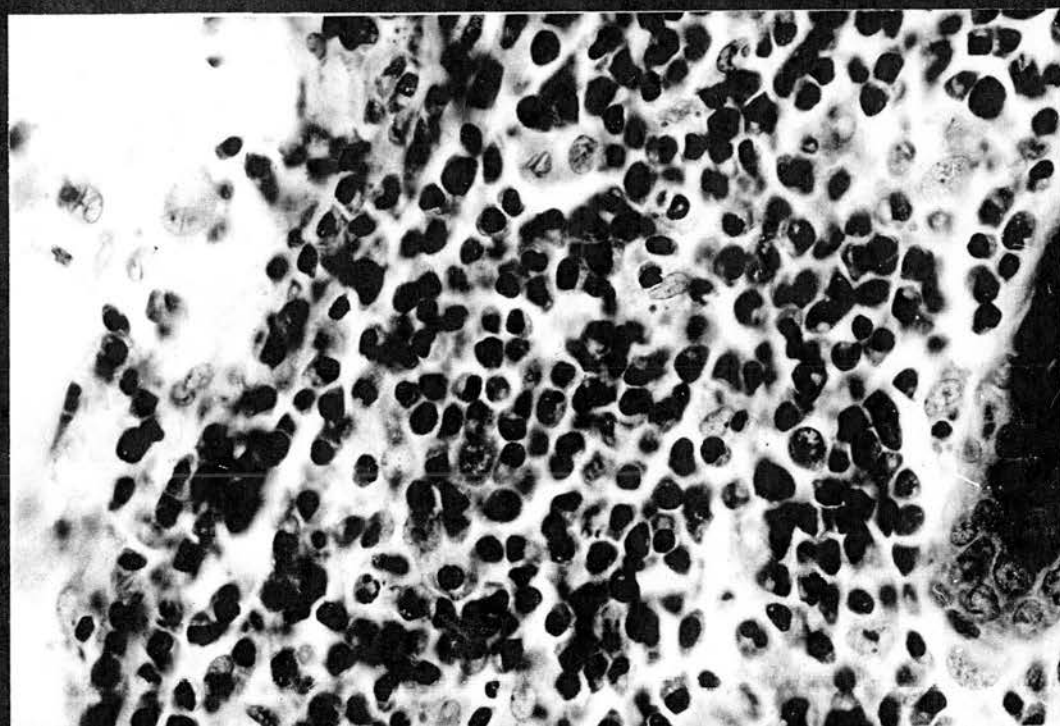
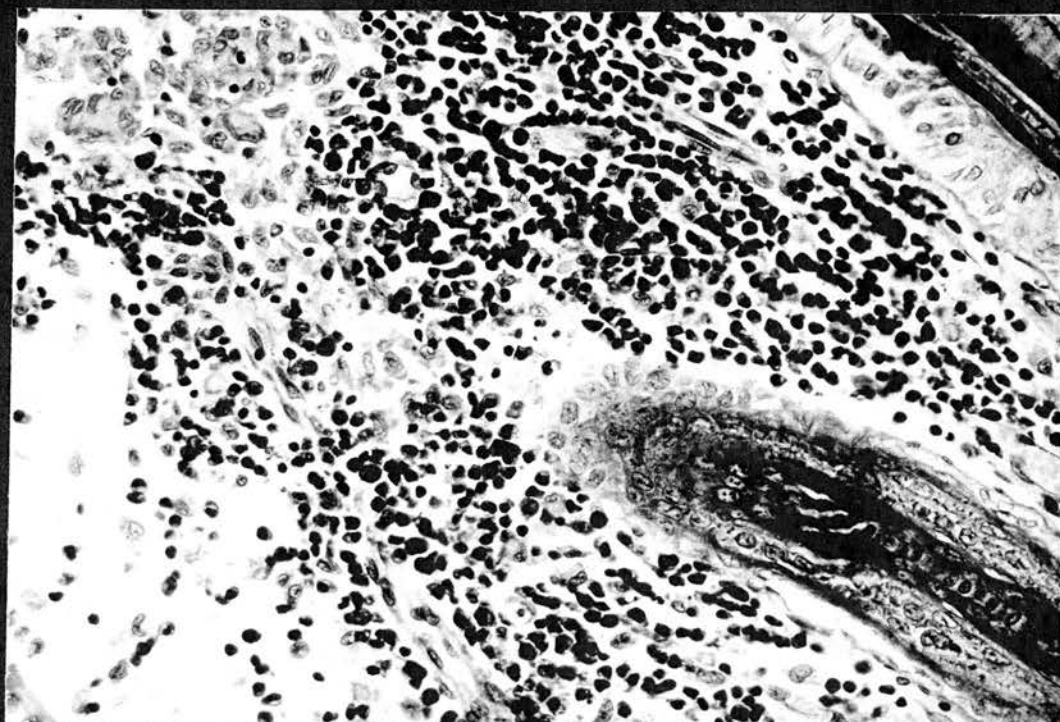


Plate 5.6A Higher magnification of papillary dermis shown in Plate 5.5B above. Note preponderance of plasma cells and absence of trypanosomes.  
(Giemsa - colophonium x 312)

Plate 5.6B Higher magnification of papillary dermis shown in Plate 5.6A above.  
(Giemsa - colophonium x 625)







### 5.5 Dispersive Action of Diminazene Aceturate on Trypanosomes in Chancres

Histological sections of 5-12 day chancres removed 2-4 days after treatment failed to reveal trypanosomes in situ (Section 5.2 above). An attempt was therefore made to determine if diminazene aceturate acts on trypanosomes in the chancres by causing them to disperse into the blood circulation.

Experimental design: Two rabbits, 1207 and 1208, were each infected with T. congolense TREU 1690 by bites from six infected tsetse flies and chancre development monitored up to days 8 and 9 respectively when they were treated. In both rabbits, the parasitaemia on day 8 was checked by microhaematocrit/buffy coat technique. Rabbit 1207 was treated with 14 mg/kg diminazene aceturate. Blood collected from an ear vein was examined at five minute intervals from 0-60 minutes post-treatment and then every 15 minutes for the next hour, every 30 minutes for the third hour and thence every hour up to six hours after treatment. At three hours after treatment, the chancre was punctured with a sterile hypodermic needle and a thin smear prepared from the expressed chancre fluid. Twenty-four hours after treatment the rabbit was killed to provide histological samples. Rabbit 1208 was treated with 14 mg/kg diminazene aceturate on day 9 after infection and blood monitored as described previously up to three hours after treatment when the animal was killed to provide histological samples.

Results: Six chancres developed on rabbit 1207 and four on rabbit 1208. The microhaematocrit/buffy coat technique revealed that trypanosomes were present in the blood of rabbit 1207 just before

treatment on day 8 post-infection. Thirty minutes after treatment a wet smear preparation revealed one trypanosome per 40 HPFs. At all other times extensive search of 40 HPFs failed to reveal trypanosomes.

No trypanosomes were found in the blood of rabbit 1208 by the microhaematocrit/buffy coat technique before treatment and it remained negative after treatment as judged by the wet smear method, up to three hours after treatment when it was euthanised for histological studies.

Histological findings on chancres from these two rabbits have already been reported in Section 5.2. Plates 5.3 and 5.4 show that numerous intact trypanosomes were present at three hours after treatment and degenerating trypanosomes could be seen 24 hours after treatment.

## 5.6 Prophylactic Effects of Diminazene Aceturate

### Experimental design

Experiment 1: Four to 16 G. m. morsitans infected with T. congolense TREU 1690 were fed on each of six pairs of rabbits 3, 6, 24, 48 or 72 hours or 7 days after treatment with 7 mg/kg diminazene aceturate. Three other pairs of rabbits were bitten by 2-4 similarly infected G. m. morsitans at 3, 6 or 24 hours after treatment with 14 mg/kg diminazene aceturate. The rabbits were examined for up to 21 days after infection for evidence of chancre development. Parasitaemia was checked by the microhaematocrit/buffy coat technique twice a week from day 14 up to day 28 after infection and the presence or absence of trypanosomes confirmed by subinoculation of blood into mice.

Experiment 2: Nine rabbits were divided into batches of three animals. In each batch one animal was left untreated as a control while the others were treated with diminazene aceturate at 7 mg/kg or 14 mg/kg. A batch of rabbits was then inoculated with  $1 \times 10^6$  cultured metacyclic trypanosomes 6, 24 or 48 hours after treatment. The inoculation of the groups was carried out on different occasions with different harvests of cultured metacyclics of T. congolense TREU 1457 separated from non-infective forms as described previously.

Experiment 3: Three pairs of rabbits were treated with diminazene aceturate 24, 6 or 3 hours prior to inoculation of cultured metacyclics of T. congolense TREU 1457. Rabbits in each pair were treated with either 7 mg/kg or 14 mg/kg diminazene aceturate. The two rabbits treated with diminazene aceturate 24 hours previously, were inoculated with  $1 \times 10^6$  cultured metacyclics of T. congolense intravenously. A control untreated rabbit was similarly inoculated with  $1 \times 10^6$  cultured metacyclics of T. congolense. The other treated rabbits were each inoculated with  $1 \times 10^6$  cultured metacyclics intradermally. A control untreated rabbit was similarly inoculated with  $1 \times 10^6$  cultured metacyclics intradermally.

After challenge, all animals were monitored for the presence of trypanosomes in the blood by the microhaematocrit/buffy coat technique, twice a week from 7-28 days after challenge. The absence of trypanosomes was confirmed by subinoculation of blood in 0.5 ml amounts into each of three mice.

### Results

In experiment 1, the six pairs of rabbits treated with 7 mg/kg diminazene aceturate at different times prior to challenge were susceptible to cyclical challenge by tsetse flies infected with T. congolense as early as three hours after treatment. Chancres and parasitaemia developed in all animals but in those challenged three or six hours after treatment only a proportion of the infective fly bites led to development of chancres (Table 5.7).

In the three other pairs cyclically challenged after treatment with 14 mg/kg diminazene aceturate, chancres appeared at bite sites and trypanosomes appeared in blood of the two rabbits challenged 24 hours after treatment while in the two pairs challenged at three or six hours after treatment, no parasitaemia developed although rabbits 1132 and 1134 challenged at three and six hours after treatment respectively, developed one and four chancres respectively (Table 5.8).

In experiment 2 in which rabbits were challenged by intravenous inoculation of cultured metacyclics, parasitaemia developed in the control untreated rabbit 1074 and in rabbits 1136 and 1139 challenged at 48 hours after treatment with 7 and 14 mg/kg diminazene aceturate respectively (Table 5.9A). In rabbits 1133 and 1128, challenged at 24 hours after treatment with 7 mg/kg and 14 mg/kg diminazene aceturate no parasitaemia developed after treatment. Unfortunately the corresponding untreated control rabbit 1071 did not develop parasitaemia. Rabbits 1130 and 1131 challenged at six hours after treatment with 7 mg/kg and 14 mg/kg diminazene aceturate respectively did not develop parasitaemia but the corresponding control untreated rabbit 1119 developed parasitaemia after challenge.

In experiment 3 in which rabbits were challenged by intravenous or intradermal inoculation of culture metacyclics, trypanosomes were not detected in the blood of rabbits 1199 and 1200 challenged intravenously 24 hours after treatment with 7 mg/kg and 14 mg/kg diminazene aceturate respectively. The corresponding untreated control rabbit 1186 developed parasitaemia after challenge. Similarly, the control untreated rabbit 1192 challenged by intradermal inoculation, developed parasitaemia after challenge. Rabbit 1187 challenged by intradermal inoculation six hours after treatment with 7 mg/kg diminazene aceturate developed parasitaemia after treatment, while rabbit 1188 which had been treated at 14 mg/kg dose rate did not develop parasitaemia following challenge six hours after treatment. Neither rabbit 1190 nor rabbit 1191 challenged three hours after treatment with 7 mg/kg or 14 mg/kg diminazene aceturate developed parasitaemias (Table 5.9B).

Table 5.7

Development of parasitaemia in rabbits challenged with Trypanosoma congolense TREU 1690 by infective tsetse bites at different times after treatment with 7 mg/kg DIMINAZENE ACETURATE.

Rabbit Number	Time of Challenge Post-treatment	Number of chancres/Number of tsetse flies fed	Presence of Trypanosomes after challenge
982	7 days	10/16	+
986	7 days	10/16	+
1080	72 hours	2/2	+
1083	72 hours	5/5	+
1079	48 hours	4/4	+
1082	48 hours	5/5	+
1078	24 hours	4/4	+
1081	24 hours	4/4	+
1123	6 hours	7/12	+
1124	6 hours	1/12	+
1120	3 hours	2/12	+
1122	3 hours	5/12	+

Table 5.8

Development of parasitaemia in rabbits challenged with Trypanosoma congolense TREU 1690 by infective tsetse bites at different times after treatment with 14 mg/kg DIMINAZENE ACETURATE.

Rabbit Number	Time of Challenge Post-treatment	Number of chancres/Number of tsetse flies fed	Presence of Trypanosomes* After challenge
1135	24 hours	3/4	+
1138	24 hours	3/4	+
1134	6 hours	4/4	0
1137	6 hours	0/4	0
1129	3 hours	0/4	0
1132	3 hours	1/2	0

\*0 = No trypanosomes detected by mouse subinoculation

Table 5.9

Development of parasitaemia in rabbits challenged with  $1 \times 10^6$  culture metacyclics of *Trypanosoma congolense* TREU 1457 at different times after treatment with DIMINAZENE ACETURATE.

## A. Group 1 rabbits challenged intravenously

Rabbit Number	Drug Dose (mg/kg)	Time of Challenge Post-treatment (hours)	Presence of Trypanosomes* After challenge
1074	-	48	+
1136	7	48	+
1139	14	48	+
1071	-	24	0
1133	7	24	0
1128	14	24	0
1119	-	6	+
1130	7	6	0
1131	14	6	0

## B. Group 2 rabbits challenged intravenously or intradermally

Rabbit Number	Drug Dose (mg/kg)	Time of Challenge Post-treatment (hours)	Inoculation Route	Presence of Trypanosomes* After challenge
1186	-	-	Intravenous	+
1199	7	24	Intravenous	0
1200	14	24	Intravenous	0
1192	-	-	Intradermal	+
1187	7	6	Intradermal	+
1188	14	6	Intradermal	0
1190	7	3	Intradermal	0
1191	14	3	Intradermal	0

\*0 = No trypanosomes detected by mouse subinoculation



### 5.7 Prophylactic Effect of Isometamidium Chloride

Studies were also carried out to determine the prophylactic effect of isometamidium chloride against cyclically transmitted T. congolense TREU 1690. Animals were treated with isometamidium chloride 4 mg/kg body weight, equivalent to 1 mg/kg in cattle as calculated by relating the dose to body surface area (Moore, 1909; Bushby, 1963).

#### Experiment 1

Experimental design: Initially 12 rabbits (Group I) were examined for development of chancres and establishment of infection following infective tsetse bites 5-8 weeks after treatment with isometamidium chloride. This was followed by similar studies on another nine rabbits (Group II) treated 1-4 weeks before challenge.

Animals in Group I were challenged in sets of three at 35, 42, 48 or 54 days after treatment. In each set of animals challenged, one rabbit was challenged by 2-4 flies, another by 6-8 flies and the third by 12-16 flies. This was intended to check whether the duration of isometamidium prophylaxis depends on the number of infected tsetse flies used in challenge.

In Group II three rabbits were challenged with 16 infected G. m. morsitans seven days after treatment and the rest of the animals in this group were challenged in pairs with 3-11 infected G. m. morsitans per animal, on days 14, 21 or 28 after treatment.

Since it was not known whether or not the presence of isometamidium in blood meals affects the subsequent infectivity of G. m. morsitans which have fed on untreated animals, each infected tsetse fly was fed only once on a treated rabbit.

After treatment, rabbits were monitored daily for development of chancres for up to 56 days after challenge. The presence of trypanosomes in blood was checked three times weekly by the microhaematocrit/buffy coat technique and by subinoculation of blood in 0.5 ml amounts in each of three mice at four, six and eight weeks after challenge.

Results: Tables 5.10 and 5.11 show the development of chancres and parasitaemia in rabbits in Groups I and II respectively. It can be seen that in Group I two out of three rabbits challenged at 35 days post-treatment failed to develop a parasitaemia while only one rabbit failed to develop parasitaemia out of each of the sets of three rabbits challenged at 42, 48 and 54 days after treatment. All 12 rabbits in this group however, developed chancres (Figures 5.5-5.8). The chancres in rabbits 938, 940 and 951 which did not subsequently develop parasitaemia, were small, measuring 3-10 mm in diameter and were seen at days 7-15 after challenge in rabbits 938 and 940 and at days 11-16 after challenge in rabbit 951.

Two rabbits, 948 and 957 which did not develop parasitaemia, showed large chancres of mean maximum diameter 15 mm and 20 mm respectively. Chancres which developed on rabbit 948 persisted for a shorter period (8-13 days post-challenge) compared to those of rabbit 957 (10-28 days after challenge).

Rabbits 941, 949, 950, 952 and 954 which became infected after challenge had large chancres (maximum mean diameters 25-45 mm) which appeared and persisted for 7-50 days after challenge (Figures 5.5C, 5.6B, 5.6C, 5.7B, 5.7C, 5.8A, 5.8B and 5.8C).

In Group II (Table 5.11) rabbits 988, 989 and 990 each challenged with 16 infected tsetse flies seven days post-treatment failed to become infected. Chancres developed at five out of 16 bite sites on rabbit 989. These chancres appeared at day 8 post-challenge, reached a maximum mean diameter of 8 mm on day 9 and disappeared by day 12 (Figure 5.4A). No chancres developed on rabbits 988 and 990.

Rabbits 1084 and 1087, challenged 14 days after treatment, with 8 and 11 infected tsetse flies respectively, failed to develop chancres and trypanosomes were not detected in either animal. In rabbits 1086 and 1089 challenged with three and four infected tsetse flies 21 days after treatment, chancres appeared at day 5 post-challenge reaching mean maximum diameters of 25 and 30 mm at days 9 and 10 after challenge (Figures 5.4B and 5.4C).

Rabbit 1085, challenged on day 28 post-treatment, developed a patent parasitaemia following the appearance of a chancre at one of the sites bitten by six infected tsetse flies. The chancre appeared at day 8 post-challenge, reached a maximum diameter of 25 mm on day 11 post-infection and had disappeared by day 17 post-challenge. Rabbit 1088 challenged at the same time with six infected tsetse flies was not infected.

## Experiment 2

Experimental design: T. congolense-infected tsetse flies which had been fed once on rabbits treated 5-8 weeks previously, with isometamidium, were tested for their ability to induce chancres and infect susceptible rabbits within seven days of feeding on drug-treated rabbits. In addition, flies which had fed on isometamidium-treated

rabbits within 7-28 days after treatment were also tested for their infectivity to mice within seven days of feeding on the treated rabbit.

Results: Forty-one flies fed on susceptible rabbits on 54 occasions and caused development of chancres on 40 occasions (74%). Twenty-six flies fed on 33 mice out of which 21 became infected (64%).

Table 5.10

Development of chancres and parasitaemia in rabbits challenged with *Trypanosoma congolense* TREU 1690 by bites from infected tsetse flies 5-8 weeks after treatment with 4 mg/kg ISOMETAMIDIUM CHLORIDE.

Rabbit Number	Day of Challenge Post-treatment	Number of chancres/flies fed	Number of tsetse flies fed	Presence of Trypanosomes* After challenge	Number of rabbits with parasitaemia/ challenged
938	35	12/12		O	
940	35	1/2		O	1/3
941	35	6/6		+	
948	42	16/16		O	
949	42	7/8		+	2/3
950	42	4/4		+	
951	48	2/16		O	
952	48	4/8		+	2/3
954	48	2/4		+	
955	54	16/16		+	
956	54	4/8		+	2/3
957	54	1/4		O	

\*O = No trypanosomes detected by mouse subinoculation

Table 5.11

Development of chancres and parasitaemia in rabbits challenged with Trypanosoma congolense TREU 1690 by bites from infected tsetse flies, 1-4 weeks after treatment with 4 mg/kg ISOMETAMIDIUM CHLORIDE.

Rabbit Number	Day of Challenge Post-treatment	Number of chancres/flies fed	Number of tsetse flies fed	Presence of Trypanosomes* After challenge	Number of rabbits/Number of rabbits with parasitaemia/challenged
988	7		0/16	0	
989	7		5/16	0	0/3
990	7		0/16	0	
1084	14		0/8	0	
1087	14		0/11	0	0/2
1086	21		1/4	+	
1089	21		2/3	+	2/2
1085	28		1/6	+	
1088	28		0/6	0	1/2

\*0 = No trypanosomes detected by mouse subinoculation

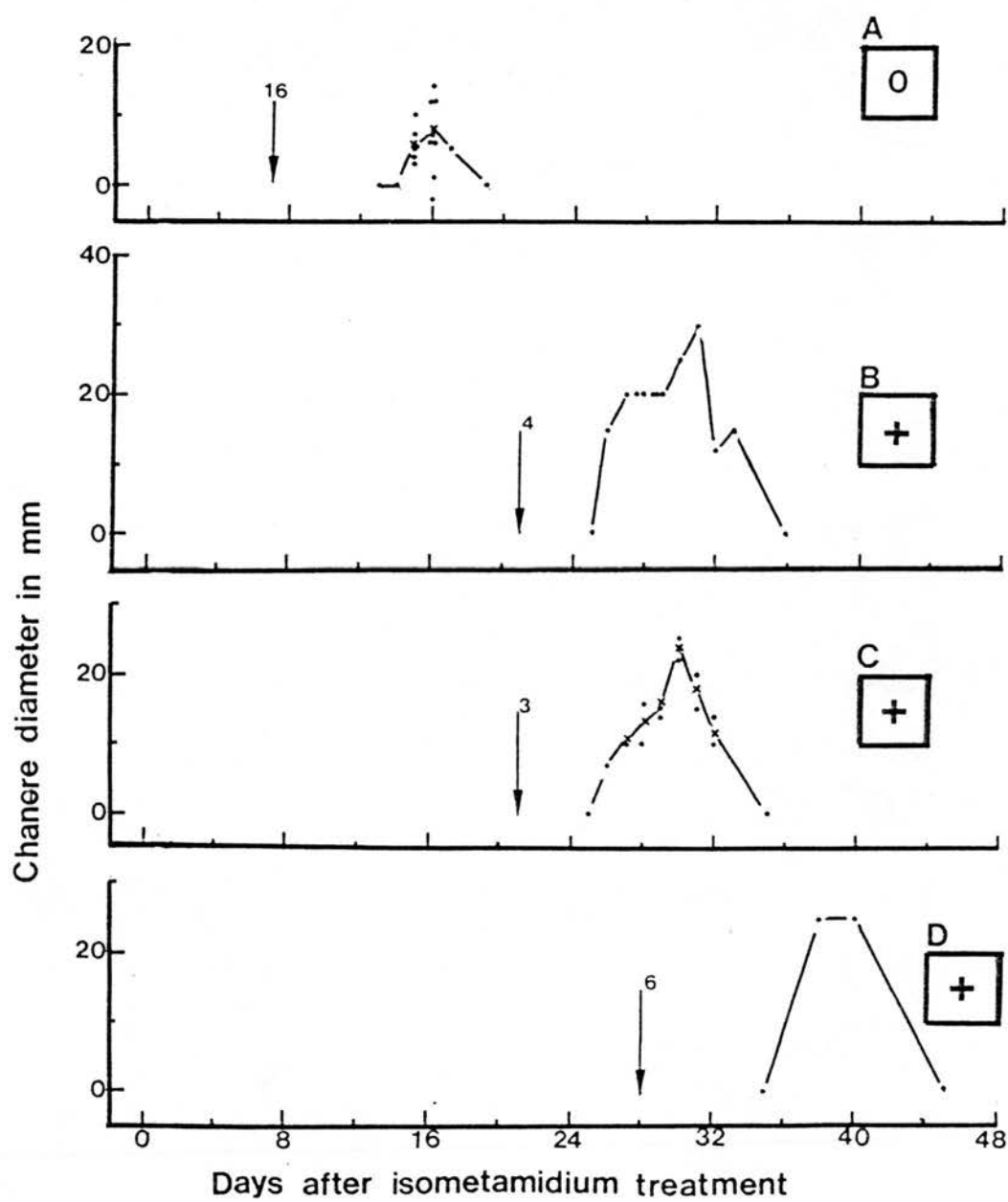
Figures 5.4 A-D Mean diameters of chancres in rabbits challenged with T. congolense TREU 1690 at different days after treatment with 4 mg/kg isometamidium chloride.

Key

A = Rabbit 989 challenged at day 7 post-treatment  
B = Rabbit 1086 challenged at day 21 post-treatment  
C = Rabbit 1089 challenged at day 21 post-treatment  
D = Rabbit 1085 challenged at day 28 post-treatment  
↓ = Bites from infected G. m. morsitans. Number of flies fed is shown on top of the arrow.

**0** = Rabbit did not develop parasitaemia after challenge

**+** = Rabbit developed parasitaemia after challenge

FIG. 5.4



Figures 5.5 A-C Diameters of chancres on rabbits challenged with T. congolense TREU 1690 35 days after treatment with 4 mg/kg isometamidium chloride.

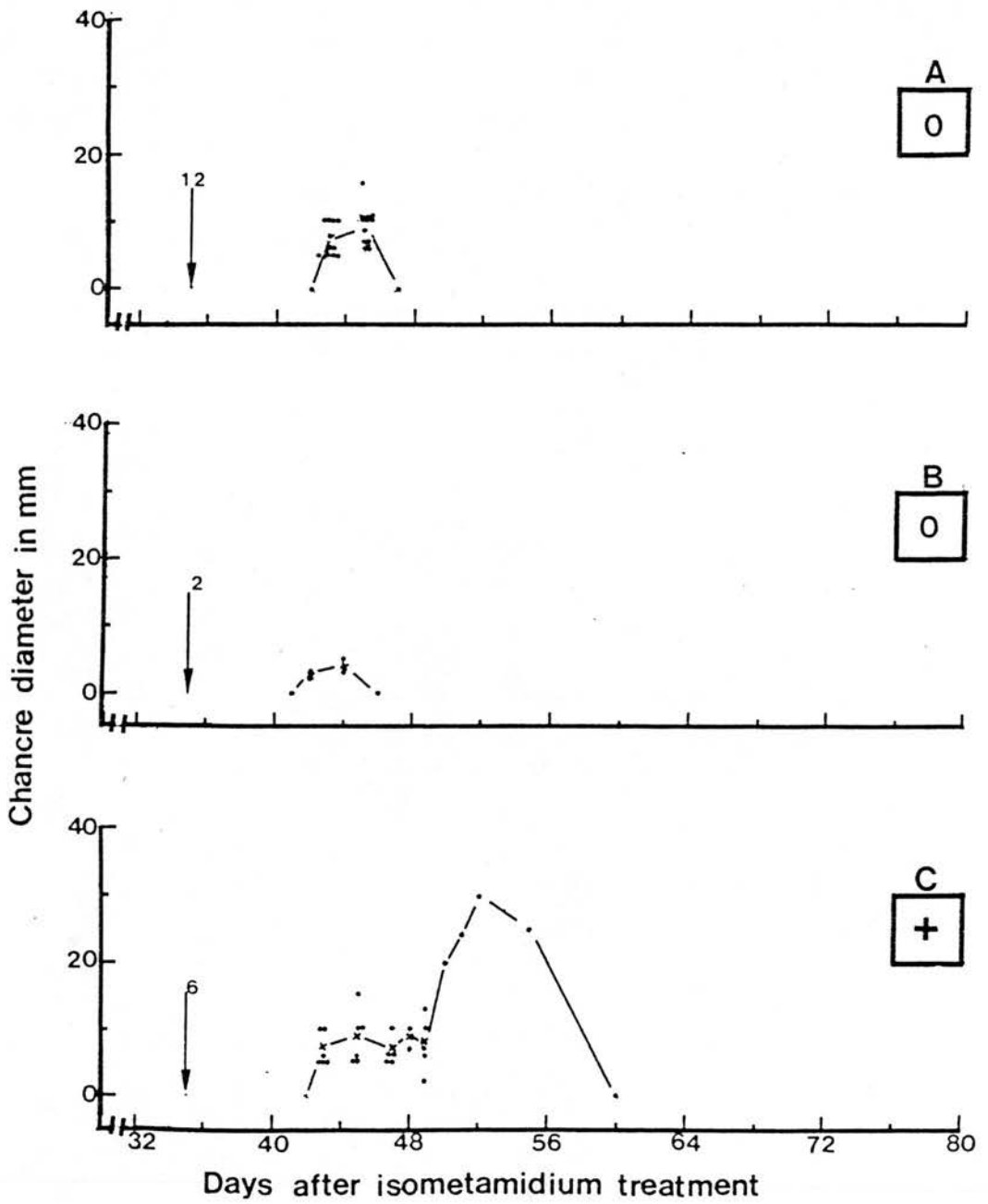
Key

A = Rabbit 938

B = Rabbit 940

C = Rabbit 941

Other symbols as shown in Figure 5.4

FIG. 5.5

Figures 5.6 A-C Diameters of chancres on rabbits challenged with T. congolense TREU 1690, 42 days after treatment with 4 mg/kg isometamidium chloride.

Key

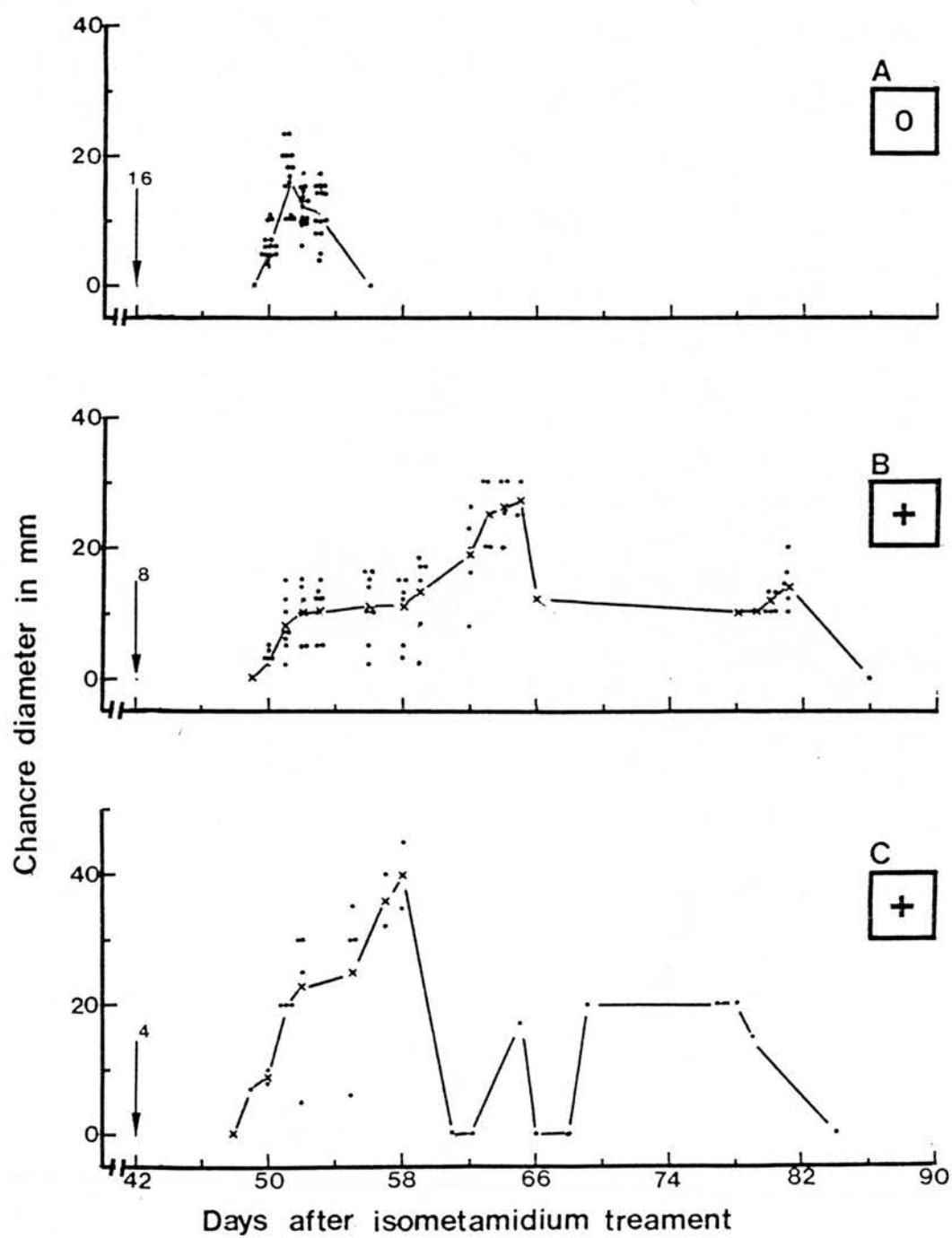
A = Rabbit 948

B = Rabbit 949

C = Rabbit 950

Other symbols as shown in Figure 5.4

FIG. 5.6



Figures 5.7 A-C Diameters of chancres on rabbits challenged with T. congolense TREU 1690, 48 days after treatment with 4 mg/kg isometamidium chloride.

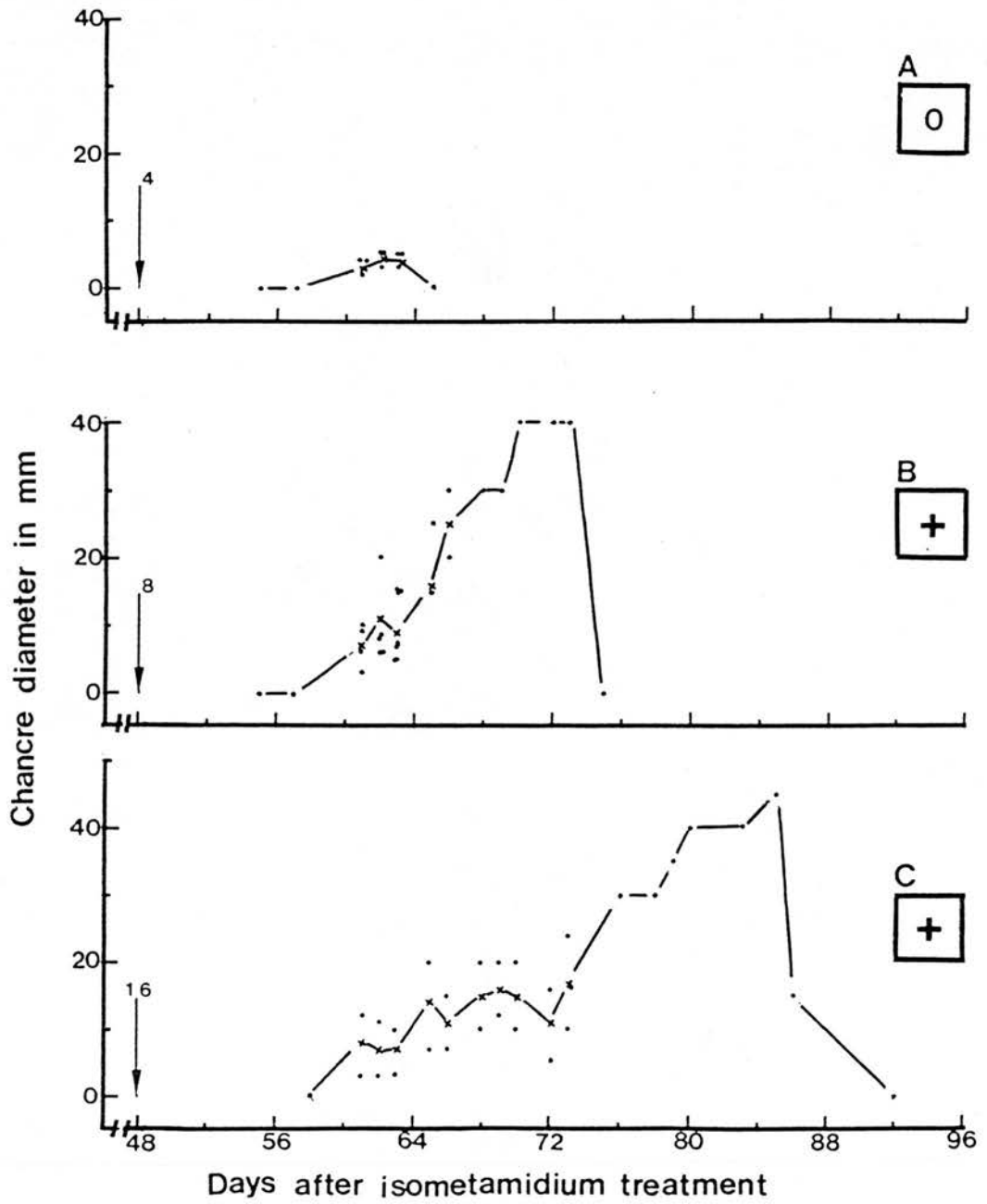
Key

A = Rabbit 951

B = Rabbit 952

C = Rabbit 954

Other symbols as shown in Figure 5.4

FIG. 5.7

Figures 5.8 A-C Diameters of chancres on rabbits challenged with T. congolense TREU 1690, 54 days after treatment with 4 mg/kg isometamidium chloride.

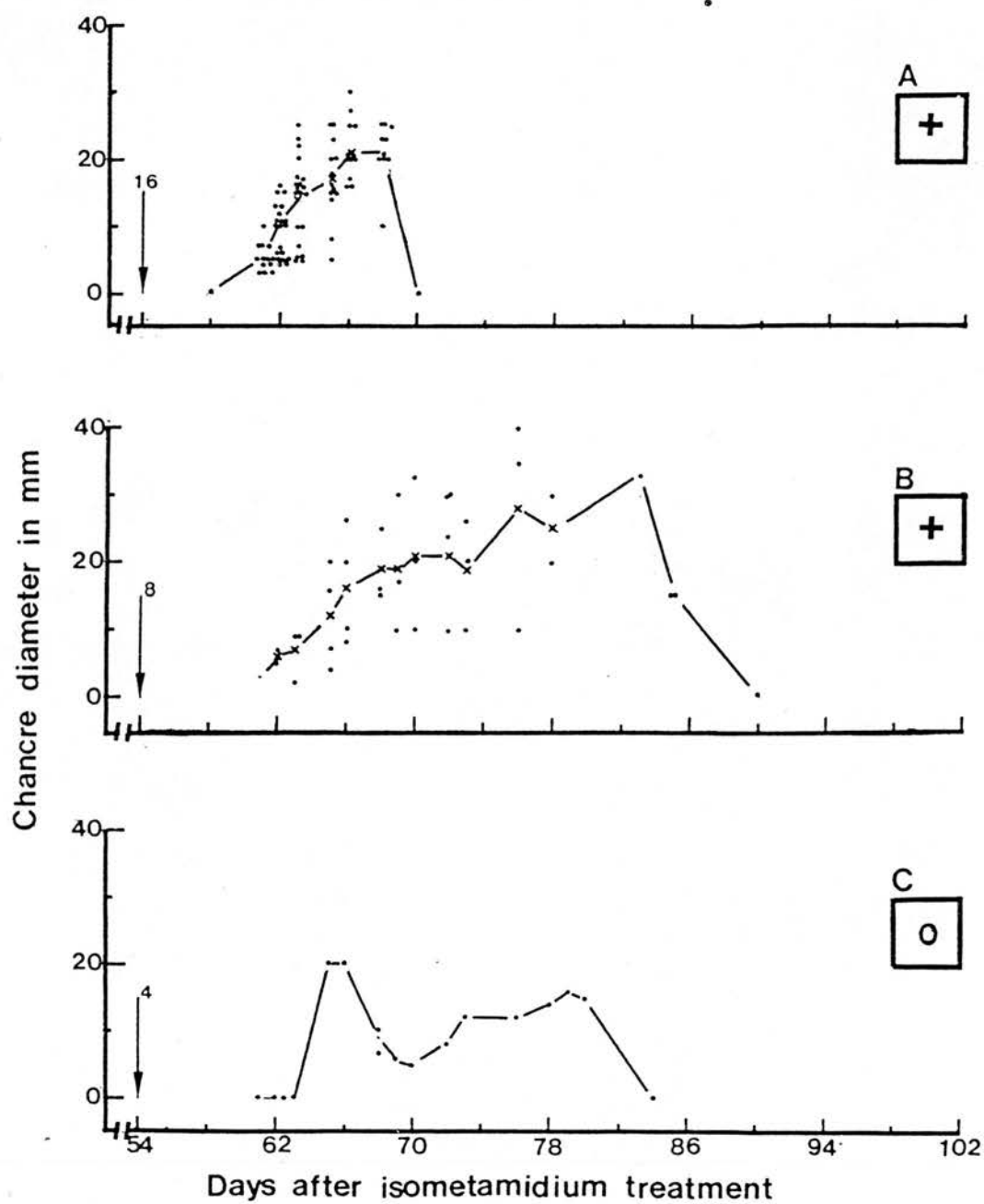
Key

A = Rabbit 955

B = Rabbit 956

C = Rabbit 957

Other symbols as shown in Figure 5.4

FIG. 5.8



## 5.8 Discussion

In studies on the development of the chancre in untreated rabbits, monitoring chancre size by measuring the diameter of associated induration and hyperaemia was found to be satisfactory and convenient in the light-skinned NZW rabbit. Other workers have monitored chancre development in goats and cattle by measuring skin thickness (Emery and Moloo, 1981; Akol and Murray, 1982). Reactions in rabbits were, however, often very turgid, which made measurement of skin thickness difficult. Moreover, it was possible that chancre development may have been interfered with due to the crushing effect of the vernier calipers used for the measurement.

The maximum diameters of trypanosome chancres in untreated rabbits were found to vary widely. This is possibly due to variations in the numbers of trypanosomes extruded by the tsetse fly as it has been shown that the size and time of appearance of chancres are often dependent on the number of metacyclic trypanosomes inoculated (Willett and Gordon, 1957; Emery et al., 1980; Luckins, Rae and Gray, 1981). The day on which the maximum diameter was reached varied between 10 and 12 days after infection. These variations in size as well as the possibility that a chancre will not appear after bites from a single infected tsetse fly (see Section 4.4) necessitated the application of several flies on rabbits used for experiments on early treatment and on prophylactic effect. On rabbit 803 treated at two days after infection for example, 12 known infected flies were fed. Feeding several infected flies at the same time on a single host did not appear to interfere with chancre development. For instance, it was demonstrated that in rabbits, bites from as many as 20 infected

flies still led to normal individual chancres. Luckins et al. (1983) also observed that local skin reactions developed at sites of each infective fly bite even after bites from as many as eight flies on the same rabbit.

Studies on the effects of treatment with diminazene aceturate on gross appearance of the trypanosome chancre, showed that early chemotherapy at two days post-infection prevented the appearance of the local skin reaction. Treatment 5-8 days after infection usually had the effect of reducing the maximum chancre size reached, although in one animal treated eight days post-infection, chancre development appeared to be unaffected.

Histological studies showed that in chancres removed 2-4 days after treatment carried out 3-10 days after infection, trypanosomes cannot be detected, although the cellular reaction seen is similar to that in sections from untreated animals at a similar period after infection. There was no evidence of in situ phagocytosis of trypanosomes. It was thus postulated that diminazene aceturate either kills chancre trypanosomes in situ or "flushes" them into the bloodstream to be phagocytosed by the reticulo-endothelial system. There have been previous suggestions that diminazene aceturate functions by presenting trypanosomes to the phagocytic immune system (Maxie and Losos, 1977) and it has been shown that in mice trypanosomes are phagocytosed in the liver (MacAskill et al., 1980).

In one rabbit (1207) in which trypanosomes could already be detected in blood, only a transient parasitaemia was detectable by examination of wet blood films, 30 minutes after treatment with diminazene acturate. In another rabbit (1208) no parasites were detectable

before or after treatment at nine days post-infection, although chancres removed from this rabbit three hours after treatment showed numerous trypanosomes within the collagen fibres. Hence it appears that diminazene aceturate does not exert its activity against chancre trypanosomes by "flushing" them into the general circulation. Since histological studies on a chancre removed from rabbit 1207, 24 hours after treatment showed degenerating trypanosomes, it would appear that trypanosomes are killed in situ.

Results from experiments on efficacy of diminazene aceturate treatment indicate that the presence of a chancre at the time of treatment may be associated with the occurrence of trypanosome relapses after treatment. Initial studies, in which the efficacy of diminazene aceturate at 7 mg/kg was assessed by attempting to isolate trypanosomes from the chancre 2-4 days after treatment, revealed that the chancre is unlikely to act as a source of relapsing trypanosomes. This may have been due to the insensitivity of the technique used to isolate trypanosomes since it was not even possible to detect parasites in a rabbit treated with a subcurative dose of homidium chloride. However, the technique was sensitive enough to detect trypanosomes in chancres from an untreated rabbit at four days after infection, at which stage the trypanosome concentration is possibly greater than that residual in a treated animal. In later experiments in which animals were monitored for appearance of relapse parasitaemia up to 42 days after treatment, it was found that such relapses occurred in animals which had been treated 8-10 days after infection when chancre size was maximal.

It is difficult to explain why relapse infections occurred only in animals treated at 8-10 days. Since animals thus treated did not invariably show relapse parasitaemia, the existence of tissue forms of trypanosomes less susceptible to the trypanocidal drug cannot be implicated. Ormerod (1979) has argued that in the case of T. brucei a tissue form different from that occurring in the blood is unlikely to occur in the chancre.

There is a possibility that the relapses occurring in animals treated 8-10 days after infection are related to the fact that trypanosome concentration in the chancre is highest between 8-13 days post-infection. Histological studies in untreated animals revealed numerous trypanosomes, particularly 8-10 days after infection. This was in agreement with observations by others (Gray and Luckins, 1980; Emery and Moloo, 1981; Akol and Murray, 1982). It has also been suggested that in a situation where the trypanosome concentration is high, the trypanocidal drug may be absorbed to such an extent that the remaining drug concentration is insufficient to effect a cure (Hawking, 1963a). This would apply to treatment with diminazene aceturate if, as suggested by Hawking (1958), this drug is absorbed in large amounts by trypanosomes. Studies with radiolabelled drugs have shown that ethidium is bound to trypanosomes - up to 79% of total radio-activity being detected as bound to T. congolense (Gilbert and Newton, 1982a).

It would possibly be expected that the small numbers of T. congolense metacyclics deposited at the bite site (ILRAD, 1982) would easily be eliminated by residual amounts of diminazene aceturate remaining in the animal after treatment, since even at 3.5 mg/kg

diminazene aceturate has been detectable for up to seven days in rabbit tissue fluid and blood (Gilbert and Newton, 1982b; Gilbert, 1983). In fact, no prophylactic effect could be detected even three hours after treatment with 7 mg/kg diminazene aceturate. Doubling the dose to 14 mg/kg only marginally increased the duration of prophylaxis to six hours after treatment.

It was shown that rabbits treated with 7 or 14 mg/kg diminazene aceturate could not be infected by intravenous inoculation of  $1 \times 10^6$  culture metacyclics 24 hours after treatment, while an untreated control rabbit became infected. Similar duration of prophylaxis has been observed in rabbits challenged with  $1 \times 10^6$  bloodstream forms of T. congolense following treatment with 3.5 mg/kg diminazene aceturate, a dose level which was shown to be subcurative for established T. congolense infection in the rabbit (Gilbert and Newton, 1983). It is possible that a slightly longer prophylactic effect is exerted by the drug, when the challenge trypanosomes are inoculated intravenously. In the present studies, a rabbit treated with 7 mg/kg diminazene aceturate could be infected with T. congolense by intradermal inoculation of  $1 \times 10^6$  culture metacyclics six hours after treatment. This is unexpected, since pharmacokinetic studies using radiolabelled drugs have shown that three hours to seven days after treatment tissue fluid levels of diminazene aceturate are higher than in blood.

The finding that diminazene aceturate at either 7 or 14 mg/kg dose rates had prophylactic effects of up to only 24 hours, is in agreement with previous findings that diminazene aceturate has little prophylactic effect against T. congolense in mice (Fink and Dann, 1974;

Nantulya et al., 1980b; Whitelaw, 1982) and is in contrast to the observed longer prophylactic effect against T. brucei sspp. in mice, rats and monkeys (Lumsden et al., 1965; Raether et al., 1972; 1974; Fink and Dann, 1974; Zahalsky and Weinberg, 1976; Whitelaw, 1982). However, in cattle Wellde and Chumo (1983) have reported that diminazene aceturate also has considerable prophylactic effect against T. congolense. Pairs of cattle could not be infected with  $1 \times 10^5$  bloodstream forms of T. congolense, two and six days after treatment with 7 mg/kg diminazene aceturate while two out of four cattle similarly treated could not be infected with a similar infective dose of T. congolense 12 days after treatment. The discrepancy between these findings and those from the present studies in rabbits could be due to host species differences as well as the use of different stocks and doses of T. congolense. It is however of great interest to determine whether diminazene aceturate in cattle also exerts considerable prophylactic effect against cyclically transmitted T. congolense.

Results obtained in the studies on prophylactic effect of isometamidium were inconsistent. There was little variation in the number of rabbits protected between three to eight weeks after treatment with 4 mg/kg isometamidium chloride. There were variations in the number of flies used for challenging individual rabbits, but whether a rabbit developed parasitaemia or not did not appear to correlate with the challenge "dose". In some cases, challenge with four infected flies led to the development of parasitaemia while challenge with 16 infected flies did not lead to development of parasitaemia. Previous observations suggested that the duration of

trypanocidal drug prophylaxis in cattle is inversely proportional to the tsetse challenge (Whiteside, 1962). Flies were fed only once on isometamidium treated rabbits so that variability of results cannot be due to reduced infectivity of flies already fed on treated rabbits.

Rabbits could be infected by cyclical challenge with T. congolense 21 days but not at 7 or 14 days after treatment with 4 mg/kg isometamidium. Since this dose was the calculated equivalent rabbit dose to the prophylactic dose of 1 mg/kg in cattle, and it had been shown that 0.75 mg/kg dose is curative in rabbits (Section 4.2.2) the results contrast with field reports that in cattle, isometamidium chloride has a prophylactic effect of at least 10 weeks (Robson, 1962; Kirkby, 1964; Wiesenhutter et al., 1968). This could be due to host species differences as well as the use of different diagnostic methods, since in the field reports there are no indications that inoculation of blood into mice to detect subpatent infections was carried out.

Studies on infectivity of flies after they had fed on isometamidium-treated rabbits showed that a high proportion of flies were still infective. Seventy-four percent of flies tested for chancre induction were positive and 64% of flies tested for infectivity to mice were positive. This is in contrast to reports that G. m. morsitans fed as teneral on a T. congolense-infected goat and then maintained on an isometamidium-treated goat, had infection rates of only 14% compared to 46% in flies maintained on a control untreated goat (ILRAD, 1983). However, in this case, flies were fed repeatedly on the isometamidium-treated animal compared to the single feed allowed in the present studies.

## CHAPTER SIX

STERILE IMMUNITY AFTER CHEMOTHERAPY OF CYCLICALLY  
TRANSMITTED TRYPANOSOMA CONGOLENSE INFECTIONS6.1 Introduction

Numerous investigations in both the field and in the laboratory have shown that trypanosomes are antigenically complex. The infected host harbours organisms which present an array of different antigens even during the course of infections initiated with a single trypanosome (Gray and Luckins, 1976; Murray and Urquhart, 1977). In the field, within any single locality animals are likely to be exposed to numerous serodemes of different species of African trypanosomes. This is possibly why there are so few reports on the development of anti-trypanosomal immunity in cattle exposed to natural infections.

The complexity of the trypanosomal antigens involved in stimulating protective immunity has prevented the development of vaccines against African trypanosomiases. However, studies on antigenic variation have attracted considerable interest not only in exploring the possible development of trypanosomal vaccines but also in providing basic knowledge of host-parasite relationships. A number of reviews covering the fields of antigenicity, immunity and host-parasite relationships of pathogenic trypanosomes have been produced in the past decade (Weitz, 1970; Desowitz, 1970; Seed, 1974; de Raadt, 1974; Gray and Luckins, 1976; Terry, 1976; Doyle, 1977; Vickerman, 1974; 1978; Holmes, 1980; Vickerman et al., 1980; Vickerman and Barry, 1982).

6.1.1 Antigens of salivarian trypanosomes

The earliest observations on antigenic variation in trypanosomes are those of Franke (1905) who found that trypanosomes in the blood of



a monkey infected with T. equinum were not affected by antibodies in the serum from the same animal and concluded that trypanosomes are liable to change immunologically during infection. Massaglia (1907) suggested that in experimental trypanosome infections of rodents each parasitaemic remission was due to the destruction of trypanosomes by host antibodies and each recrudescence in parasitaemia was due to proliferation of parasites with a different antigenic constitution to their predecessors. In man, early observations on the relapsing course of parasitaemia in trypanosome infections were reported by Ross and Thomson (1910) who made a daily record of parasite numbers in a sleeping sickness patient undergoing treatment.

Antigenic variation is a highly efficient way in which parasites may ensure survival in the immunologically intact host but there are various other ways in which parasites may survive. Schistosoma spp., for example, are known to exhibit concomitant immunity whereby the immune response has little effect on established adult worms although it can act against the invading Schistosomula of new infections. This evasion of the immune response by adult worms is due to host-like antigenic determinants found on the surface membranes of the schistosomes which block attachment of antibodies directed against the parasite (Clegg, 1974; Smithers and Doenhoff, 1982).

Trypanosomes have also been shown to acquire host protein on their surfaces but it is not known whether this has immunologically protective function in the natural hosts. Thus, although there is evidence for the binding of host protein by a T. vivax strain adapted to rodents, no host-protein binding by T. vivax in the natural ungulate

host has been reported (Vickerman, 1974). In addition, De Gee and Rovis (1981) failed to demonstrate host protein on a tsetse-transmissible, mouse-infective strain of T. vivax. Diffley and Honigberg (1977, 1978) showed, by means of quantitative immunofluoresence, that in T. congolense infections in rats, the parasites acquire host protein on their surface. They did not however proceed to show whether or not the acquisition of host protein interferes with antibody attachment to the surface of the trypanosome.

Variation in antigens of trypanosomes during the course of infection has been shown to be associated with a surface glycoprotein coat covering the whole of the trypanosome, including the flagellum. This compact, 12-15 mm thick coat was first described in ultrastructural studies of T. brucei, T. congolense and T. vivax (Vickerman, 1969a,b). It is seen in bloodstream forms and in metacyclic forms from the tsetse fly but is absent from trypanosomes found in the midgut of the insect vector and similar developmental stages obtained by in vitro cultivation (Vickerman, 1969a). It has been argued that the surface coat in T. brucei and T. congolense bloodstream forms is unlikely to represent host protein since a similar coat is present in metacyclics within the tsetse fly (Vickerman, 1974).

Evidence that antigenic variation is associated with change in the surface coat has been summarised by Turner (1982). Firstly, procyclic trypomastigotes which have no surface coat are incapable of antigenic variation and assume a common surface antigenicity irrespective of the antigenic variant from which they were derived (Seed, 1964). Secondly, the loss of surface coat is paralleled by loss of the ability of variant specific antibodies to agglutinate the trypanosomes

(Barry and Vickerman, 1979). Thirdly, variant specific antibodies have been shown by electron microscopy to bind to the surface coat of homologous but not heterologous trypanosomes (Vickerman and Luckins, 1969). Lastly, Cross (1975) has purified a single variable surface glycoprotein from the trypanosome surface membrane and used this to produce variant specific antisera which reacted with the surface coat of homologous but not heterologous trypanosomes.

The number of variable antigen types (VATs) which can be produced by salivarian trypanosomes is a subject of much research interest. Various serological tests have been used for VAT identification. The agglutination test has been widely used in the case of T. brucei organisms (Cunningham and Vickerman, 1962; Gray, 1962; 1965a,b; Capbern et al., 1977). The neutralization of infectivity test was found more suitable for T. congolense (Wilson and Cunningham, 1972; Uilenberg and Giret, 1972), while the immunolysis test has been applied in the case of T. vivax (Dar, 1972; Jones and Clarkson, 1972). Early studies indicated that a clone of T. brucei could produce at least 15-23 VATs (Ritz, 1916; Osaki, 1959; Gray, 1965b). Recently a clone of T. equiperdum has been shown to produce 101 VATs (Capbern et al., 1977), and it is possible that there is no limit to the number of antigens which could be produced by a clone of T. brucei other than that imposed by the time for which the infected host survives (Gray, 1965b).

There are problems in attempting to assess the full capacity of a strain to produce different antigens since in the serological tests used, although trypanosome populations may react as discrete serological entities with homologous antisera, the population may consist of

trypanosomes of several different antigen types. Hence, the antigenic character of a relapse variant can result from a combination of the characteristics of several different antigens (Gray and Luckins, 1976). Indirect fluorescent antibody tests utilising monoclonal antibodies have enabled a more comprehensive determination of individual antigenic types comprising mixed populations of trypanosomes (Hajduk and Vickerman, 1981).

There is a tendency for trypanosomes of any single serodeme to revert to a limited number of characteristic VATs whenever they are transmitted cyclically. Early studies on T. brucei indicated that with each strain, populations of trypanosomes transmitted by different tsetse flies were more closely related to each other than to the variants the flies originally ingested (Broom and Brown, 1946). This observation has been confirmed in a number of subsequent studies using T. brucei sspp. (Gray and Luckins, 1976). The term "basic antigen" has been applied to the antigen which appeared to be common between cyclically transmitted trypanosomes (Gray, 1965a).

The existence of a basic antigen in a T. congolense stock has also been demonstrated (Uilenberg and Giret, 1972). Trypanosomes isolated by mouse subinoculation from sheep during a short febrile period before patent parasitaemia had what was considered as the basic antigen. Wilson and Cunningham (1970) however, failed to demonstrate the existence of a basic antigen in T. congolense. In their study, stabilates used in neutralization tests were prepared from mice infected for 17-20 days. During this time antigenic variation may have taken place. More recent studies have shown that cyclical transmission of different VATs of a stock of T. congolense in mice results in metacyclic

populations which are similar in VAT composition as judged by immunofluorescence and neutralization of infectivity tests (Nantulya et al., 1980a).

Evidence that metacyclic trypanosomes bear a basic antigen was provided by Cunningham (1966) who prepared populations of metacyclics for neutralization test by allowing T. rhodesiense-infected flies to feed on pools of blood. Sera collected from sheep within 14 days of infection contained neutralizing antibodies against the metacyclic trypanosomes. Some flies were shown to transmit metacyclics of more than one antigenic type. Similarly, Gray (1965a) found that although some flies transmitted only the "basic antigen" others transmitted a mixture of "basic" and other variable antigens. Hajduk and Vickerman (1981) using immunofluorescence and trypanolysis tests, provided further evidence that a tsetse fly ingesting a particular VAT of T. brucei may transmit several metacyclic VATs. From these findings it was concluded that serodemes of trypanosomes are cyclically transmitted as repertoires of metacyclic VATs.

In addition to the existence of basic antigen, there are antigens which tend to appear predictably in the early stages of infection in each new host whenever a stock is passaged from host to host by syringe. These have been termed "predominant antigens" (Gray, 1962).

The mechanism of induction of antigenic variation is not known with certainty (Gray and Luckins, 1976; Vickerman, 1978). It is widely held that antigenic variation is genetically controlled but mutation, which could account for the variation, can be ruled out on account of the predictable appearance of some antigenic types (Gray, 1965a). There is evidence that antigenic variation results from alternate

gene expression rather than changes in the genes themselves (Vickerman, 1978).

#### 6.1.2 Immunity to salivarian trypanosomes

Animals suffering from African trypanosomiasis produce large amounts of trypanosomal antibodies but their presence is not correlated with immunity. There is a pronounced hypergammaglobulinaemia (Kobayashi and Tizard, 1976; Luckins and Mehltz, 1976; Masake et al., 1983), and while it was initially shown that some of the immunoglobulins may be non-specific for trypanosomes (Houba et al., 1969), more recent evidence has shown that antibodies produced are specific to the infecting organisms (Musoke et al., 1981; Masake et al., 1983).

Studies on antigenic variation in African trypanosomiasis have made vaccination against trypanosomiasis appear to be an unattainable goal. However, it must be remembered that most of the studies have involved serological tests, results of which may or may not correlate with protective immunity. Weitz (1970) has stressed the importance of deliberate infection with live organisms to demonstrate development of immunity against trypanosomiasis. Laboratory and field studies involving deliberate infections or exposure to natural challenge have indicated that both laboratory and domestic animals acquire immunity to trypanosomiasis, although it appears to be variant specific.

Several procedures have been used to induce active immunity against trypanosomiasis (Gray and Luckins, 1976; Murray and Urquhart, 1977). The most successful methods have been inoculation of live irradiated organisms and infection and treatment. In one study in which cattle were inoculated with killed trypanosomes or minimal doses of live T. brucei, T. congolense and T. vivax equivocal results were obtained

when the animals were exposed to field challenge (Schilling, 1935; Hornby, 1941). Half the number of animals so treated survived in an enzootic area (Schilling, 1935). However, when the same animals were moved to a different area with adverse conditions and heavier trypanosome challenge, they succumbed to trypanosomiasis (Hornby, 1941).

Attempts have been made to induce immunity to T. congolense in cattle, dogs and mice (Duxbury et al., 1972). Cattle and dogs were immunised with  $10^8$ - $10^{10}$  irradiated trypanosomes and challenged with  $10^4$ - $10^5$  trypanosomes. Mice were immunised with  $10^6$ - $10^7$  irradiated trypanosomes and challenged with  $10^3$  trypanosomes. Whereas the mice showed resistance, the cattle and dogs were fully susceptible. As it appears that the T. congolense used was an uncloned stock it is possible that the larger dose used to challenge cattle and dogs contained more antigenic variants than that used in mice.

In a subsequent experiment, Wellde et al. (1973) attempted immunisation of cattle with gamma-irradiated T. rhodesiense. Cattle which were inoculated with six immunising doses of  $10^9$ - $10^{10}$  irradiated trypanosomes showed resistance to challenge with  $10^4$  viable trypanosomes at one week and eight months after immunisation but not at 14 months. Animals which underwent self-cure after infection were resistant to challenge 14 months later. Infections with this stock of T. rhodesiense were mild even in control cattle. However, Wellde et al. (1973) did not relate the mild nature of T. rhodesiense infections in cattle, to the ease with which protective immunity could be induced as compared to induction of immunity to T. congolense (Duxbury et al., 1972).



More recently, Morrison et al. (1982a) immunised cattle with irradiated T. brucei and showed that cattle immunised with at least  $10^7$  irradiated organisms were immune to challenge with  $10^3$  infective trypanosomes 14 days later. Antibody detected by various serological tests including neutralization of infectivity, correlated with immunity. No similar studies have been carried out with T. congolense.

The earliest observations on development of immunity following chemotherapeutic treatment of trypanosomal infections were probably those of Bevan (1928, 1936) who noted that clinical cases of bovine trypanosomiasis which recovered after treatment frequently remained in good health despite reinfection. Whiteside (1962) reported a lengthening of the intervals between the development of new infections in cattle treated with diminazene aceturate while under moderate field challenge. Higher challenge resulted in a decrease in the interval between the appearance of overt parasitaemia. Fiennes (1970) noted that after three years of chemotherapy a herd of beef cattle showed few cases of trypanosomiasis.

The trypanocidal drug regime used has been found to be important in development of immunity. Animals treated with diminazene aceturate upon development of clinical disease (or a PCV of 20%) have been shown to develop partial immunity (Wilson et al., 1975, 1976). In contrast, in the same study, animals treated as a group with diminazene aceturate whenever patent parasitaemia developed in any one animal in the group, did not develop immunity.

Sterile immunity has been claimed to develop in animals under antrycide prophylaxis (Soltys, 1955). However, other studies could not confirm this, as it was shown that antrycide given repeatedly can leave



residues which can provide prophylactic effect long after treatment has ceased (Smith, 1958).

There have been few laboratory studies on the development of immunity in association with chemotherapy. Cunningham (1968) treated six cattle at 14 days after infection with T. brucei and then challenged them in pairs at one, two and three months after treatment. It was shown that such animals resisted challenge for up to three months after treatment while controls were susceptible.

Immunity has also been reported to develop after treatment of T. congolense infection in cattle (Wilson, 1971). Cattle were inoculated with two doses of T. congolense bloodstream forms at intervals of 11 weeks, each inoculation being followed by treatment with diminazene aceturate at 21 days after each infection. The five cattle were shown to be immune to challenge 22 weeks after the first immunisation. In contrast, animals which were bitten by wild-caught flies infected with T. congolense on five occasions over a period of 320 days, each infection being treated at 21 days, were still susceptible when challenged on a sixth occasion with the same metacyclics (Wilson, 1971). It was concluded that sterile immunity was achieved when immunising and challenge infection consisted of one well defined antigenic type.

In sheep, Uilenberg and Giret (1973) demonstrated that immunity can develop against metacyclics of T. congolense after treatment of cyclically transmitted infection with diminazene aceturate. One sheep treated three weeks after cyclical infection was shown to be immune to cyclical challenge with the same stock 9-15 weeks after the first infection. In another sheep similarly treated, immunity to cyclical challenge was demonstrated 9-11 weeks but not 14 weeks after first

infection. In contrast, three sheep treated 3-4 weeks after cyclical infection with T. congolense were not immune to challenge with  $10^7$  bloodstream forms isolated from 4-6 different animals cyclically infected with the same stock.

An interesting aspect of immunisation by infection and treatment of cyclically transmitted infections is whether animals develop immunity when treated during the periods when trypanosomal chancres were present but before patent parasitaemia had developed. Early treatment may not allow the full development of immunity. Field studies by Wilson et al. (1975, 1976) showed that animals treated with diminazene aceturate on a herd basis when one animal in the group became infected did not develop immunity whereas animals treated individually, after development of disease, were immune. More recently, Emery et al. (1980) reported development of immunity to homologous cyclical challenge with T. congolense in cattle treated with diminazene aceturate at day 15 after primary cyclical infection but not in those similarly treated five or ten days after primary infection. These animals were challenged at 21 days post-treatment of primary infection.

Studies on the development of immunity to cyclically transmitted T. congolense infections in rabbits following treatment with homidium chloride have also been reported (Luckins et al., 1983). In contrast to the finding of Emery et al. (1980) it was shown that rabbits treated seven days after infection were immune to homologous cyclical challenge 75 days after treatment. One out of eight rabbits and two out of four rabbits challenged at 125 and 300 days after treatment respectively were also shown to be immune. Levels of serum antibodies measured by ELISA and neutralization of infectivity were low in the treated immune

rabbits as in susceptible rabbits.

There is obviously a need for more information on the development of immunity after abrogation of cyclically transmitted T. congolense infections in both the laboratory and in the field. In the work to be described, immunity and serological responses in rabbits infected with T. congolense were studied in relation to the timing of chemotherapeutic treatment and the development of chancres.

## 6.2 The Effect of Early Chemotherapy on the Serological Response in Animals Infected with Trypanosoma congolense

Experimental design: Two rabbits were treated with diminazene aceturate after bites by G. m. morsitans infected with T. congolense TREU 1457. The development of serum antibodies was monitored by ELISA and was compared to that in an infected, untreated rabbit. Rabbits 803 and 809 bitten by 12 and 11 infected flies respectively were treated with 7 mg/kg diminazene aceturate at two and five days after infection. Rabbit 821, bitten by one infected fly, was left untreated.

Chancre development was monitored by measuring the diameter of induration and erythema at the tsetse bite sites for up to 21 days post-infection. Serum samples for estimation of anti-trypanosomal antibodies were collected at 2-3 day intervals for the first week after infection and thereafter at weekly intervals up to day 63.

Results: Rabbit 803 treated on day 2 post-infection showed no serological evidence of trypanosomiasis (Figure 6.1A). This corresponded with complete lack of chancre development at sites bitten by infected tsetse flies. No anti-trypanosomal antibodies were detected

in rabbit 809 treated at day 5 post-infection, although small chancres developed between day 6 and 11 days after the flies had fed (Figure 6.1B). In contrast, rabbit 821 bitten by one infected tsetse fly and left untreated showed high ELISA values beginning on day 14 and reaching a maximum by day 42 after infection (Figure 6.1C).

Figure 6.1 Mean diameters of chancres and mean ELISA values of serum anti-trypanosome antibodies in rabbits 803, 809 and 821 at different days after infection with T. congolense TREU 1457.

A = Rabbit 803 treated at day 2 post-infection

B = Rabbit 809 treated at day 5 post-infection

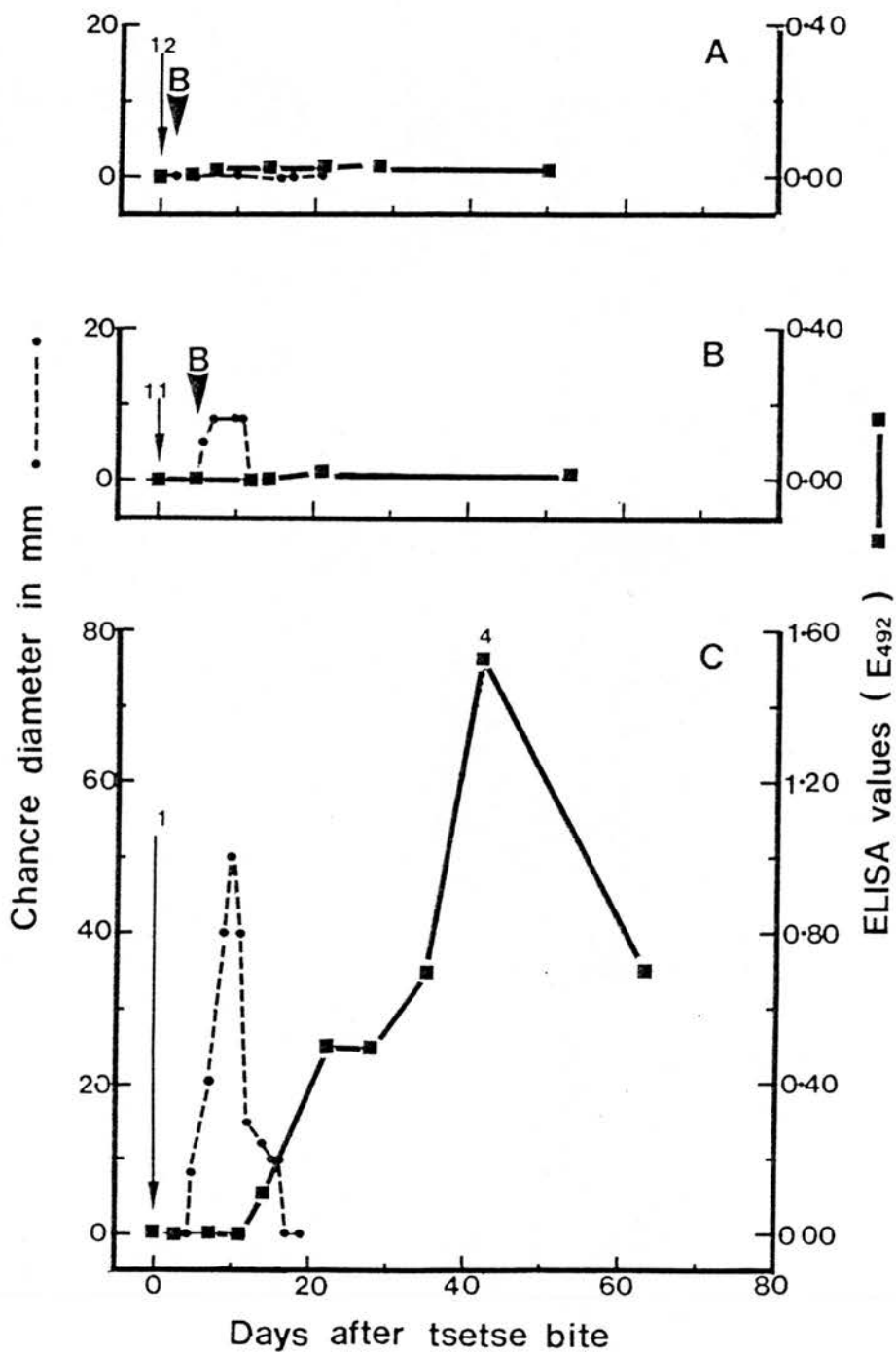
C = Rabbit 821 left untreated

**B**

▼ = Treatment with diminazene aceturate

↓

Bites from infected tsetse flies:  
Number of flies fed is shown above  
the arrow.

FIG. 6.1

### 6.3 Immunity in Rabbits Treated at Different Times after Infection with Trypanosoma congolense

Experimental design: Two groups of rabbits, Group I and II each with 14 animals were used in studies on sterile immunity following abrogation of cyclical T. congolense infections with 7 mg/kg diminazene aceturate. In the first group of animals tissue chambers were implanted to allow the collection and assay of tissue fluids.

Group I - Rabbits 896, 893, 892, 889, 886 and 887 were bitten by 1-2 G. m. morsitans infected with T. congolense TREU 1682 and then treated at 0, 4, 8, 12, 21 and 42 days post-infection respectively. Rabbits 882, 881, 880, 877, 876, 884 and 874 were bitten by 1-2 G. m. morsitans infected with T. congolense TREU 1690 and then treated at 2, 4, 6, 10, 21, 27 and 42 days after infection respectively. One rabbit, 885, was bitten simultaneously by two flies infected with T. congolense TREU 1682 and two flies infected with T. congolense TREU 1690 and then treated at day 42 after infection.

All rabbits were challenged 40-43 days post-treatment, by bites from 1-3 G. m. morsitans infected with the homologous stock of T. congolense. Rabbit 885 which had received dual primary infection was challenged with G. m. morsitans infected with T. congolense TREU 1690. Flies which were fed on rabbits which subsequently failed to develop chancres were allowed to feed on susceptible rabbits to ascertain that they were still capable of infecting animals and causing chancres. All flies used in challenge studies were less than 77 days old. Animals treated up to seven days after primary infection were challenged with the same batch of flies used in primary infection while those challenged at later times were bitten by another batch of flies newly infected

with the homologous stock of T. congolense.

Chancre development was monitored up to 21 days after either primary or challenge infection. Development of parasitaemia prior to treatment was checked by the microhaematocrit/buffy coat technique in all animals except those treated up to four days after infection. After treatment, development of parasitaemia was monitored by the microhaematocrit/buffy coat technique twice weekly from 14 days post-treatment to the day of challenge. Prior to challenge it was confirmed that each animal was not infected, by subinoculation of blood in 0.5 ml amounts into each of three mice. After challenge, development of parasitaemia was monitored by the microhaematocrit/buffy coat technique twice weekly, 14-28 days after challenge. The absence of trypanosomes in blood was confirmed by subinoculation of blood in 0.5 ml amounts into each of three mice.

Serum was collected from all animals at 2-3 day intervals up to day 14 after primary tsetse bite and thereafter at weekly intervals up to four weeks after challenge. For comparative assay of anti-trypanosomal antibodies tissue fluid was collected at 2-3 day intervals up to 14 days post-primary challenge and thereafter at weekly intervals up to the day of treatment with diminazene aceturate.

Tissue fluid was collected from tissue chambers created by implanting hair curlers subcutaneously 21 days prior to primary tsetse bite, according to methods described by Goodwin and Guy (1973). Hair curlers used were 6.0 cm long by 1.8 cm outer diameter. They were covered with a belt of silicone rubber tubing to discourage the growth of tissue over the inner surface. Implantations were carried out under halothane anaesthesia. One hair curler was implanted on each flank



(Plate 6.1). For the primary infection, tsetse flies were allowed to feed directly on top of the tissue chamber.

In the course of collecting tissue fluid, care was taken to avoid trauma which would result in contamination of the tissue fluid with blood. Fluid was withdrawn into a 1 ml syringe through a 21 gauge, 38 mm needle inserted through the silicone belt area of the hair curler. It was diluted 1:4 with sterile PBS pH 7.4 and centrifuged at 1500 g for 30 minutes to remove cellular debris and then stored at -20°C until used.

Tissue fluid and sera were assayed for anti-trypanosomal antibodies using ELISA and Neutralization of Infectivity test. For the Neutralization of Infectivity test, pooled sera and tissue fluid from rabbits 874 and 885 were used.

A local skin reaction was removed from one rabbit (879) under halothane anaesthesia, at day 9 post-infection. Half of the chancre was macerated in cold, sterile PBS (pH 7.4) and the fluid extract assayed for trypanosomal antibodies using the ELISA test.

Group II - Primary and challenge infections were made using G. m. morsitans infected with T. congolense TREU 1690. Three rabbits, 937, 975 and 971 were treated on days 7, 7 and 8 after bites from 23, 4 and 19 infected tsetse flies respectively. They were then challenged by bites from eight infected tsetse flies 42 days post-treatment. Two rabbits, 959 and 970 were treated 14 days after bites from 15 and 9 infected tsetse flies respectively and challenged with eight infected tsetse flies 42 days post-treatment. Three other rabbits, 958, 962 and 961 were treated on days 17, 18 and 20 after bites from 11, 8 and 21 infected tsetse flies and then challenged with eight infected tsetse flies 42 days post-treatment. Six rabbits numbered 939, 942, 943, 944

945 and 947, were treated at day 28 after bites from 2-5 infected tsetse flies and then challenged using 4-16 infected tsetse flies, 40 days after treatment.

All rabbits were checked for chancre and parasitaemia development as described above for Group I.

#### Results:

(a) Resistance to challenge - In group I only one animal (877) out of eight treated 0-12 days after primary tsetse bite was immune to homologous challenge. In contrast, all six rabbits treated at 21-42 days after primary tsetse challenge failed to develop parasitaemia (Table 6.1).

In group II, rabbits 937, 975 and 971, treated at 7 or 8 days after primary tsetse bite, were susceptible to homologous challenge whereas all 11 animals treated at 14-28 days after primary tsetse bite were immune (Table 6.2).

(b) Chancre development and serological reactions - Mean chancre diameters and serological reactions in rabbits from group I are shown in Figures 6.2-6.8. In the following description, chancre and antibody development in treated animals are compared to that in rabbit 821 (Figure 6.1C) which was a fully susceptible, untreated animal.

Treatment on day 0 and 2 in rabbits 896 and 882 resulted in complete suppression of the chancre development following the primary tsetse bite (Figure 6.2). After challenge, chancre development and serological reaction were similar to those of susceptible rabbits (Figure 6.1C).

When treatment was carried out four days after primary infection in rabbits 881 and 893 (Figure 6.3) there was no detectable serological

response although small chancres were observed to develop at the bite sites. Homologous challenge resulted in normal chancre development and serological reaction similar to that seen in susceptible rabbits.

Treatment at 6 and 8 days after primary infections (rabbit 880 and 892) resulted in the appearance of chancres with mean diameters similar to those of untreated rabbits but there was no indication of antibody development in these animals (Figure 6.4). On challenge, both rabbits showed an accelerated chancre development in which reactions appeared as early as day 3 post-challenge. The serological reactions were similar to that of a susceptible, untreated rabbit on primary infection (Figure 6.1).

Rabbit 877, treated 10 days post-primary infection showed an apparently normal chancre development and rising antibody titre reaching maximum levels 25 days after treatment (Figure 6.5A). However, neither microhaematocrit/buffy coat technique nor mouse subinoculation revealed the presence of trypanosomes in the blood. Following challenge, only small reactions were observed and although there was a rise in antibody levels trypanosomes were not detected in the peripheral blood.

In another instance, rabbit 889 (Figure 6.5A) treated on day 12, showed normal chancre development but no evidence of anti-trypanosomal antibodies after treatment. In homologous challenge there was accelerated chancre development and rapid rise in antibody levels.

Rabbits treated at 21-42 days after infection (876, 886, 884, 874, 887 and 885) showed normal chancre and antibody development up to the time of treatment. Thereafter, antibody levels dropped gradually (Figures 6.6-6.8). At the time of challenge, antibody levels were

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similar to or only slightly higher than pre-infection levels. After challenge, all six rabbits developed only small chancres and the antibody levels remained low. No trypanosomes were found in the blood.

(c) Antibody levels in tissue fluids and serum - The ELISA values obtained using tissue fluid and serum samples collected from rabbits 876, 886, 874, 887 and 885 are shown in Figure 6.9. In all rabbits antibody levels in tissue fluid were lower than those in serum.

The lower antibody levels in tissue fluid compared to serum were confirmed by the neutralization of infectivity test using T. congolense TREU 1725 and pooled serial sera and tissue fluid from rabbits 874 and 885 (Table 6.3). Sera collected 35, 42 and 49 days after infection showed complete neutralization of infectivity while sera collected at 21, 28, 56 and 63 days after infection showed partial neutralization. Tissue fluid on the other hand had only partial neutralizing activity at days 35, 42 and 49 after infection. No neutralizing activity could be detected in tissue fluid collected at other times. Fluid extract from a chancre removed from rabbit 879 was also negative for trypanosomal antibodies

Plate 6.1 Rabbit with implanted hair curlers and regressing  
chancres near the implantations. Sample hair  
curlers are shown on the bottom of the picture.  
The middle bulging on each implantation is due  
to a silicone rubber ring.



Table 6.1

Development of parasitaemia after homologous challenge of rabbits infected with Trypanosoma congolense TREU 1682 or TREU 1690 and treated with DIMINAZENE ACETURATE.

Rabbit Number	Number of flies used for primary infection	Day of Treatment Post-infection	Day of Challenge Post-treatment	Number of/Number of flies used chancres/ for challenge	Presence of Trypanosomes	
					Before*	After**
896	1	0	42	3/3	NC	+
882	2	2	40	2/2	NC	+
881	2	4	42	1/1		+
893	1	4	42	1/1		+
880	2	6	42	1/1	O	+
892	1	8	42	2/2	O	+
877	1	10	42	0/2		+
889	2	12	41	3/3	+	O
876	1	21	42	0/1	+	+
886	1	21	42	0/3	+	O
884	1	27	40	0/1	+	O
874	1	42	40	0/2	+	O
887	2	42	43	0/3	+	O
885	4	42	43	0/3	+	O

\*O = No trypanosomes detected by microhaematocrit/buffy coat technique

\*\*O = No trypanosomes detected by mouse subinoculation

NC = Not checked

Table 6.2

Development of parasitaemia after homologous challenge of rabbits infected with Trypanosoma congolense TREU 1690 and treated with DIMINAZENE ACETURATE.

Rabbit Number	Number of flies used for primary infection	Day of Treatment Post-infection	Day of Challenge Post-treatment	Number of/Number of flies used chancres/ for challenge	Presence of Trypanosomes	
					Before* treatment	After** challenge
937	23	7	42	7/8	0	+
975	4	7	42	6/8	0	+
971	19	8	42	6/8	0	+
959	15	14	42	0/8	+	0
970	9	14	42	0/8	+	0
958	11	17	42	0/8	+	0
962	8	18	42	0/8	+	0
961	21	20	42	0/8	+	0
939	4	28	40	0/4	+	0
942	3	28	40	0/8	+	0
943	4	28	40	0/8	+	0
944	4	28	40	0/4	+	0
945	5	28	40	0/15	+	0
947	2	28	40	0/16	+	0

\*0 = No trypanosomes detected by microhaematocrit/buffy coat technique

\*\*0 = No trypanosomes detected by mouse subinoculation



Figure 6.2 Mean diameters of chancres and mean ELISA values of serum anti-trypanosome antibodies in rabbits treated at 0 or 2 days post-primary infection with T. congolense TREU 1682 or TREU 1690.

Key

A = Rabbit 896

B = Rabbit 882



= Treatment with diminazene aceturate



= Bites from infected G. m. morsitans.  
Each arrow represents one tsetse fly which fed.

FIG. 6.2

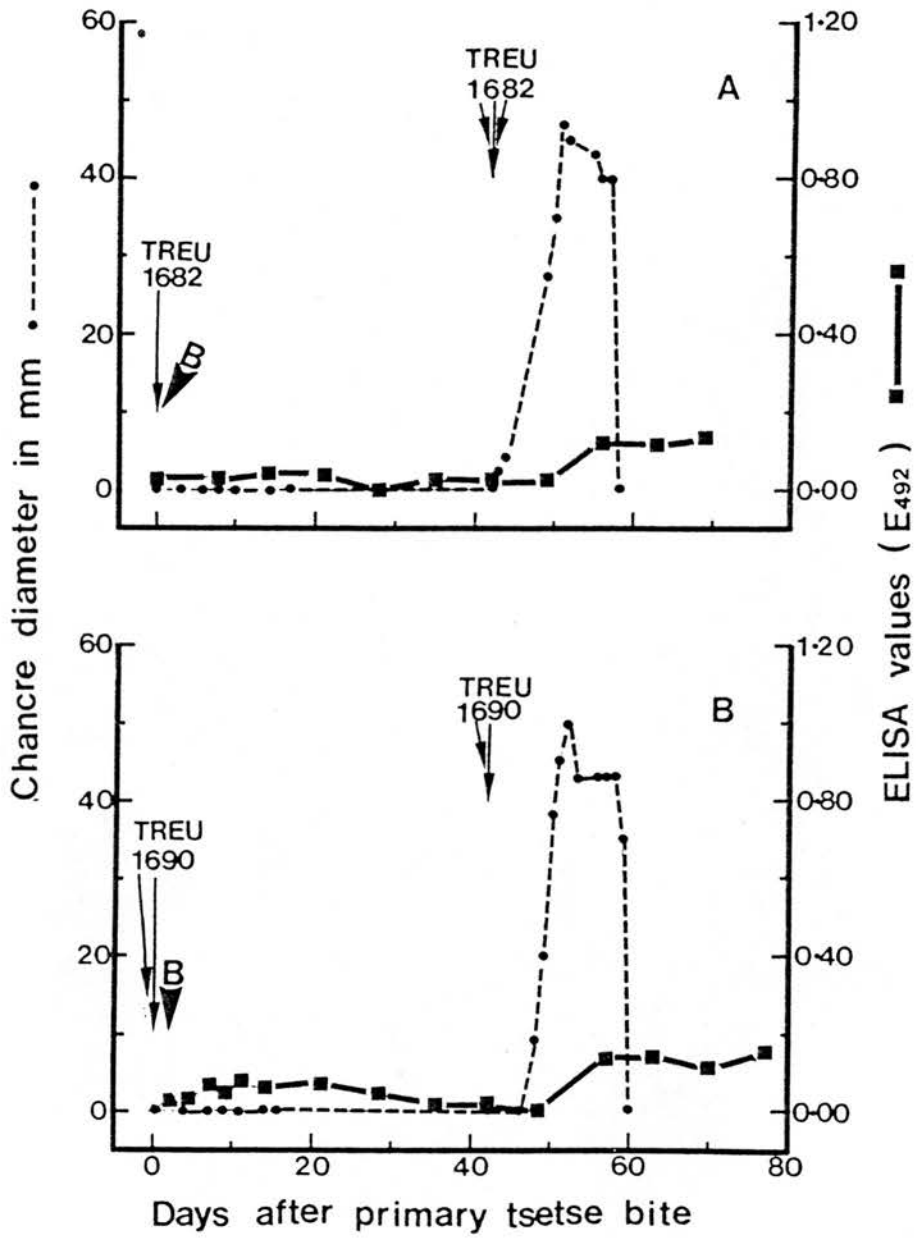


Figure 6.3 Mean diameters of chancre and mean ELISA values of serum anti-trypanosome antibodies in rabbits treated at 4 days after primary tsetse-bite, using flies infected with T. congolense TREU 1682 or TREU 1690.

Key

A = Rabbit 881

B = Rabbit 893

Other symbols as shown in Figure 6.2

FIG. 6.3

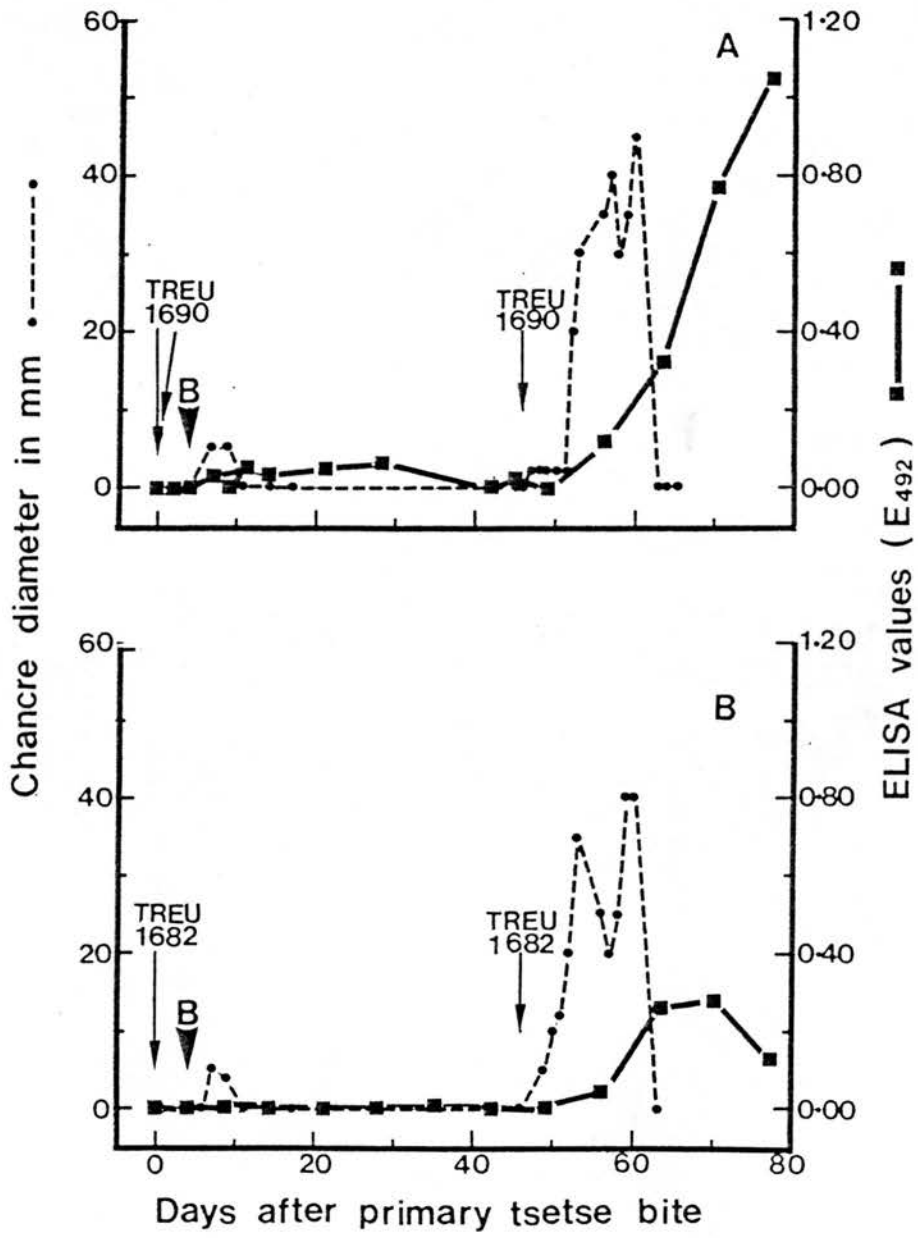


Figure 6.4 Mean diameters of chancres and mean ELISA values of serum anti-trypanosome antibodies in rabbits treated at 6 and 8 days after primary infection, using tsetse flies infected with T. congolense TREU 1682 or TREU 1690.

Key

A = Rabbit 880

B = Rabbit 892

Other symbols as shown in Figure 6.2

FIG. 6.4

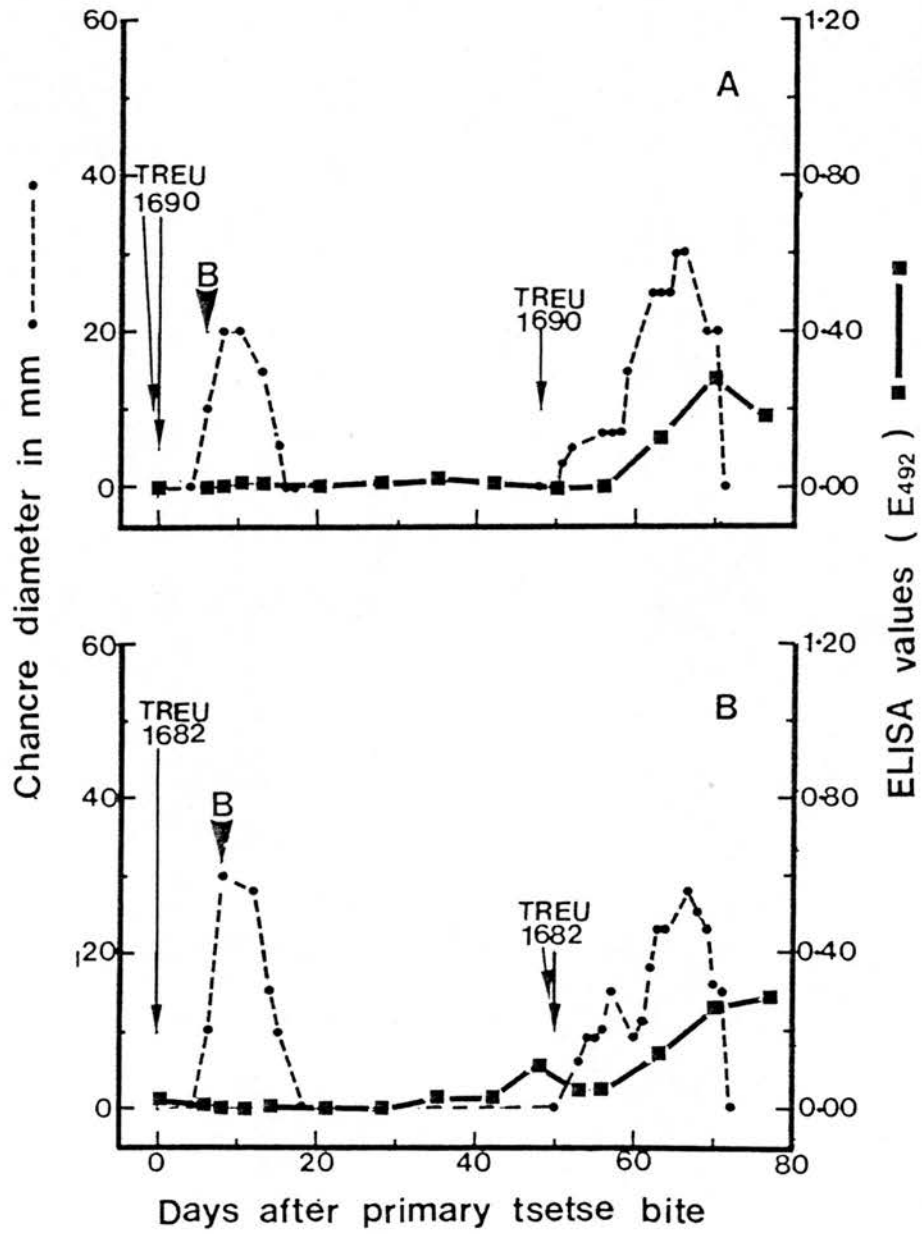


Figure 6.5 Mean diameters of chancres and mean ELISA values of serum anti-trypanosome antibodies in rabbits treated at 10 and 12 days after primary tsetse-bite with flies infected with T. congolense TREU 1682 or 1690.

Key

A = Rabbit 877

B = Rabbit 889

Other symbols as shown in Figure 6.2

FIG. 6.5

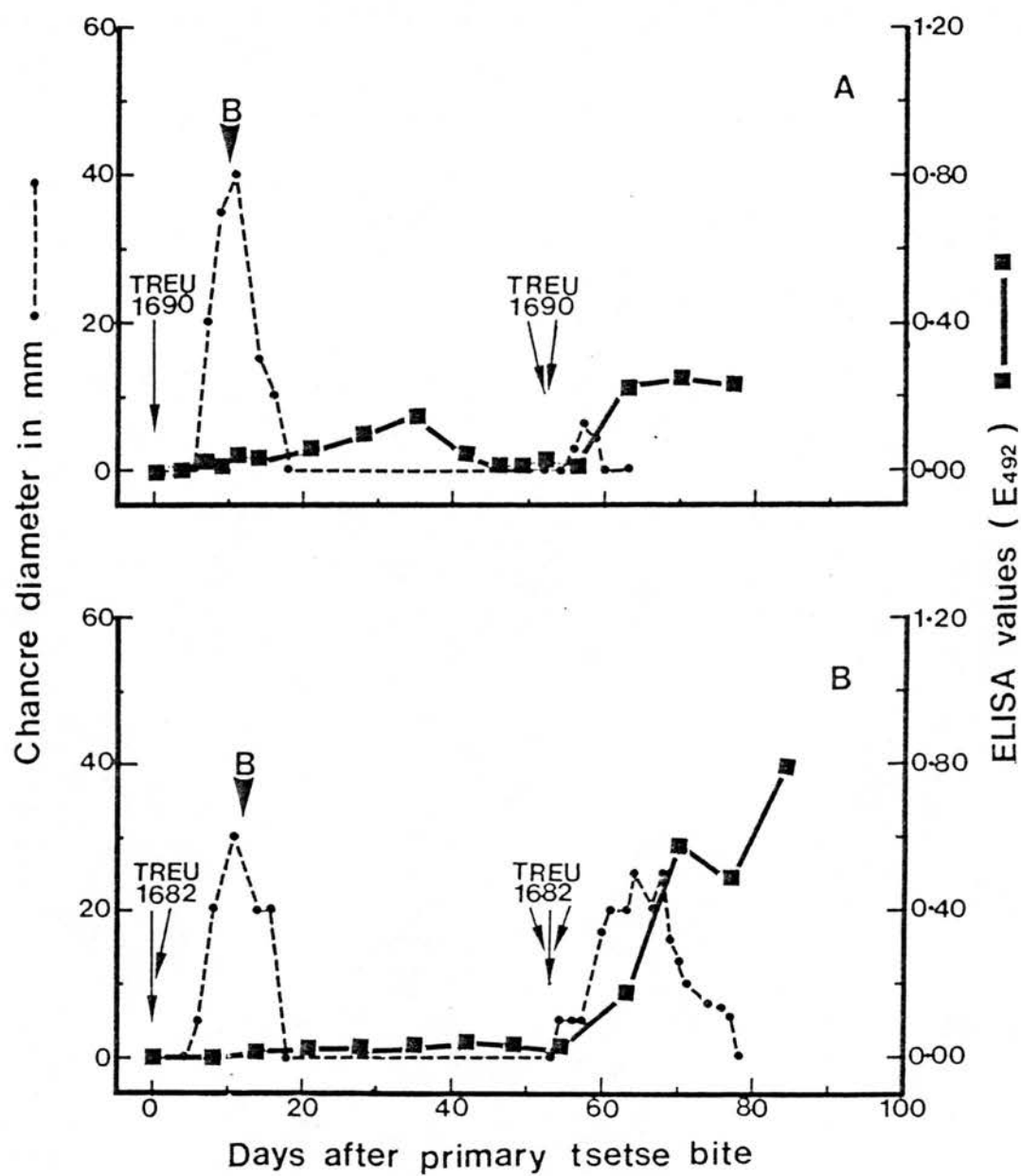




Figure 6.6 Mean diameters of chancres and mean ELISA values of serum anti-trypanosome antibodies in rabbits treated at 21 days after primary tsetse bite, using flies infected with T. congolense TREU 1682 or TREU 1690.

Key

A = Rabbit 876

B = Rabbit 886

Other symbols as shown in Figure 6.2

FIG. 6.6

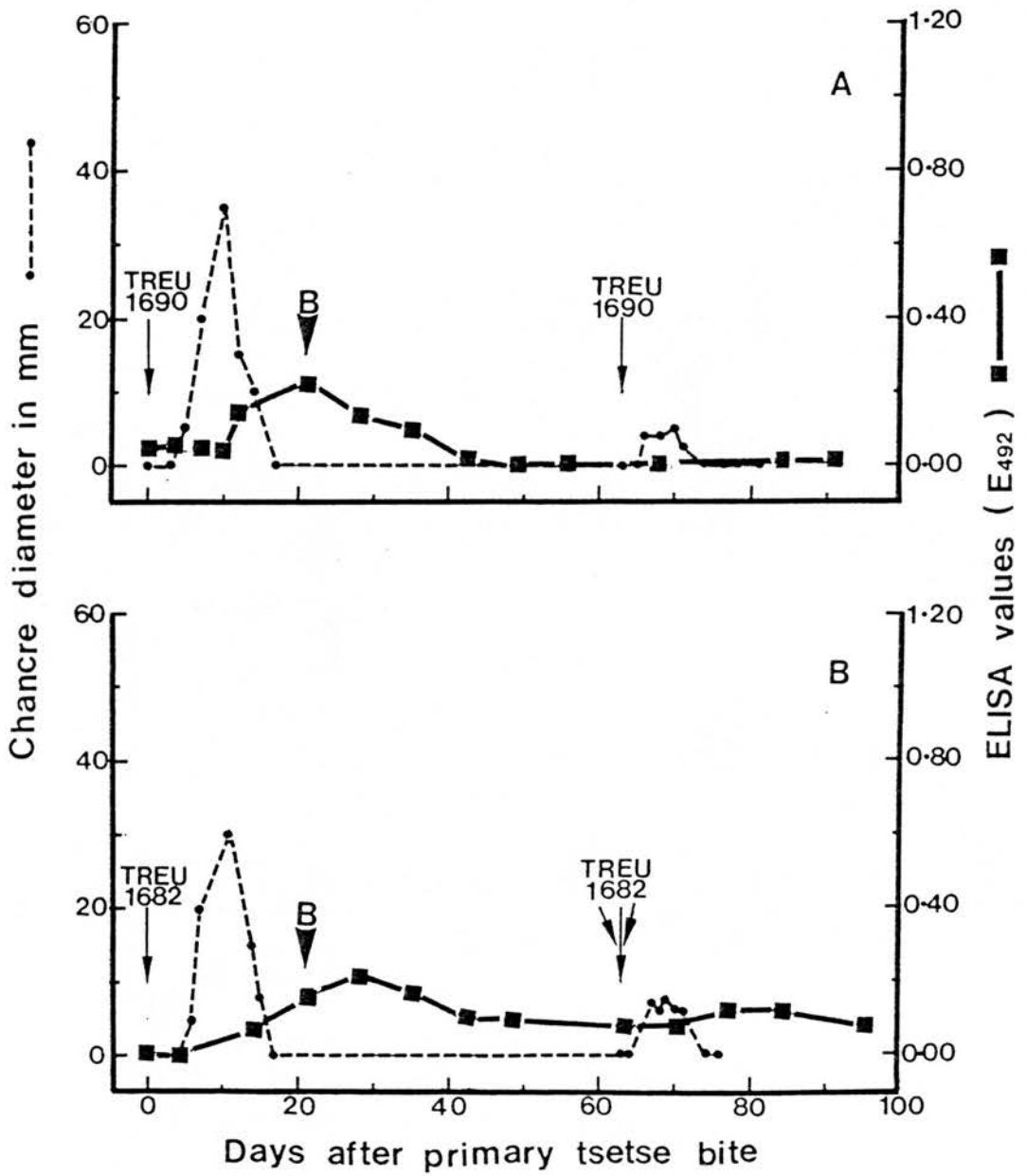


Figure 6.7 Mean diameters of chancres and mean ELISA values of serum anti-trypanosome antibodies in rabbits treated at 27 and 42 days after primary tsetse bite, using flies infected with T. congolense TREU 1690.

Key

A = Rabbit 884

B = Rabbit 874

Other symbols as shown in Figure 6.2

FIG. 6.7

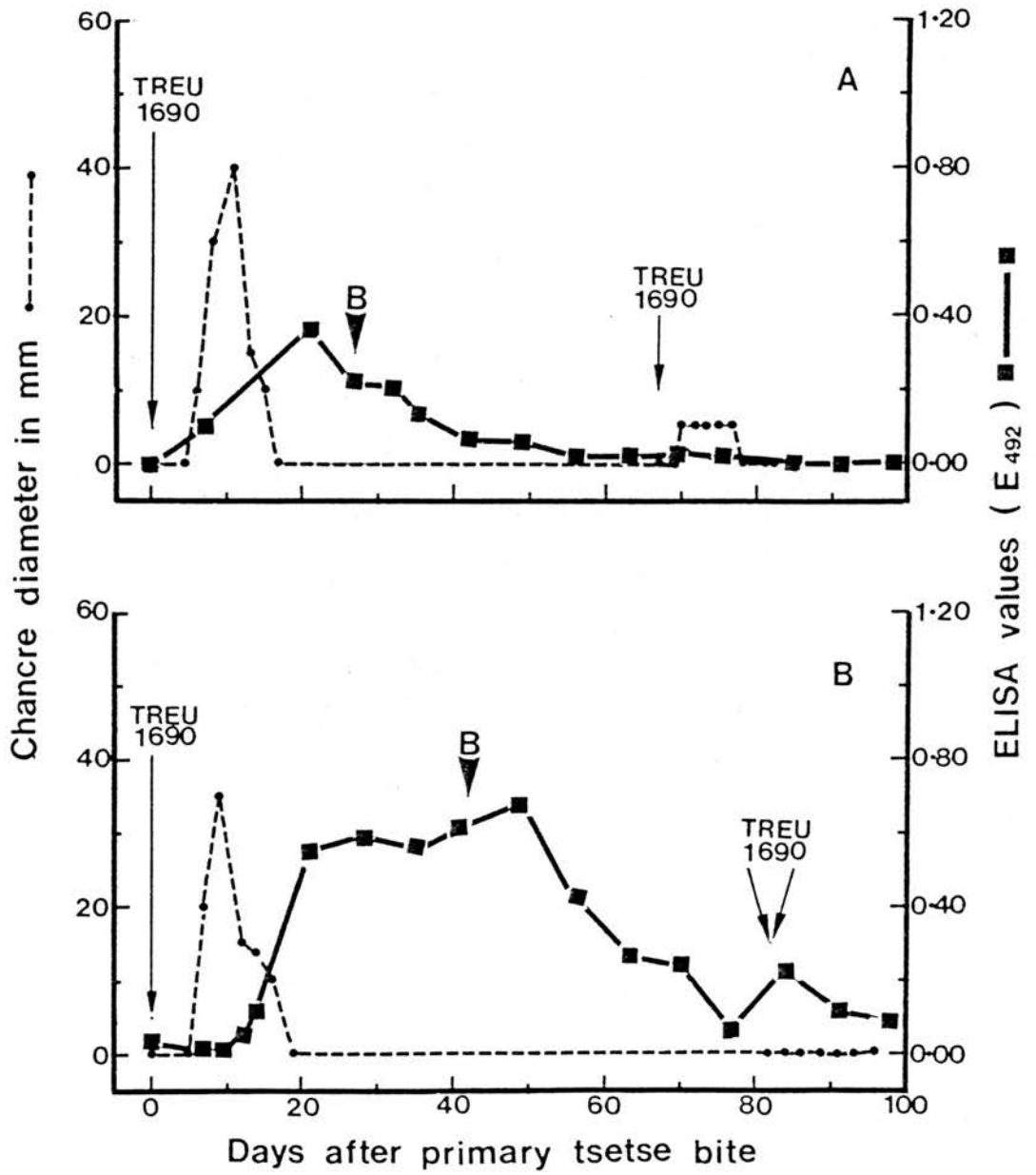


Figure 6.8 Mean diameters of chancres and mean ELISA values of serum anti-trypanosome antibodies in rabbits treated at 42 days after primary tsetse bite, using flies infected with T. congolense TREU 1682 or TREU 1690.

Key

A = Rabbit 887

B = Rabbit 885

Other symbols as shown in Figure 6.2

FIG. 6.8

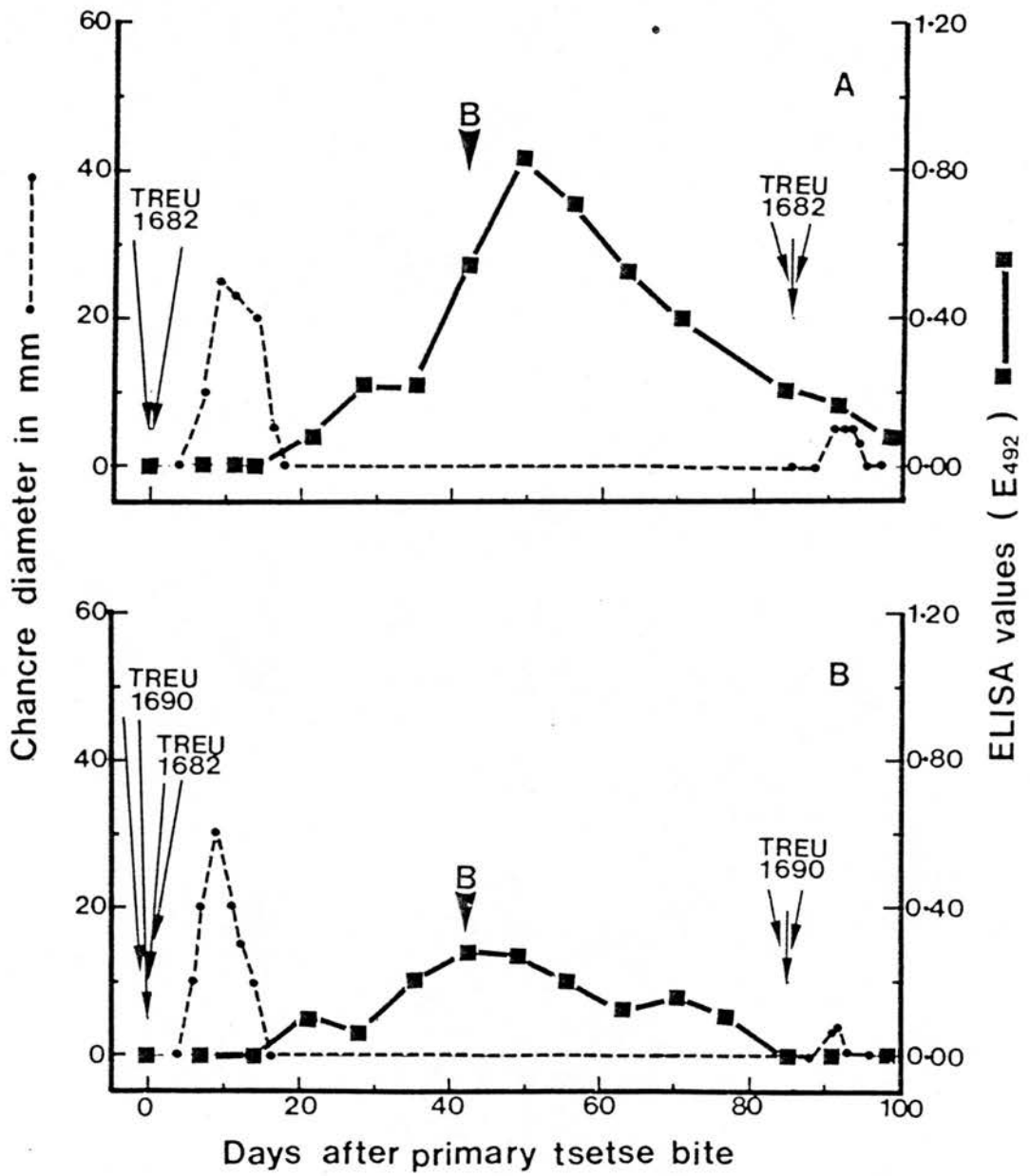


Figure 6.9 Mean ELISA values of anti-trypanosome antibodies in serum and tissue fluid from rabbits cyclically infected with T. congolense TREU 1682 or TREU 1690.

Key

A = Rabbit 876 }  
B = Rabbit 886 } Treated with diminazene aceturate  
                  } at 21 days post-infection

C = Rabbit 874

D = Rabbit 887

E = Rabbit 885

↓ = Infective tsetse bite. Each arrow represents one tsetse fly which fed.

■ — ■ = Serum

▲ — ▲ = Tissue Fluid

FIG. 6.9

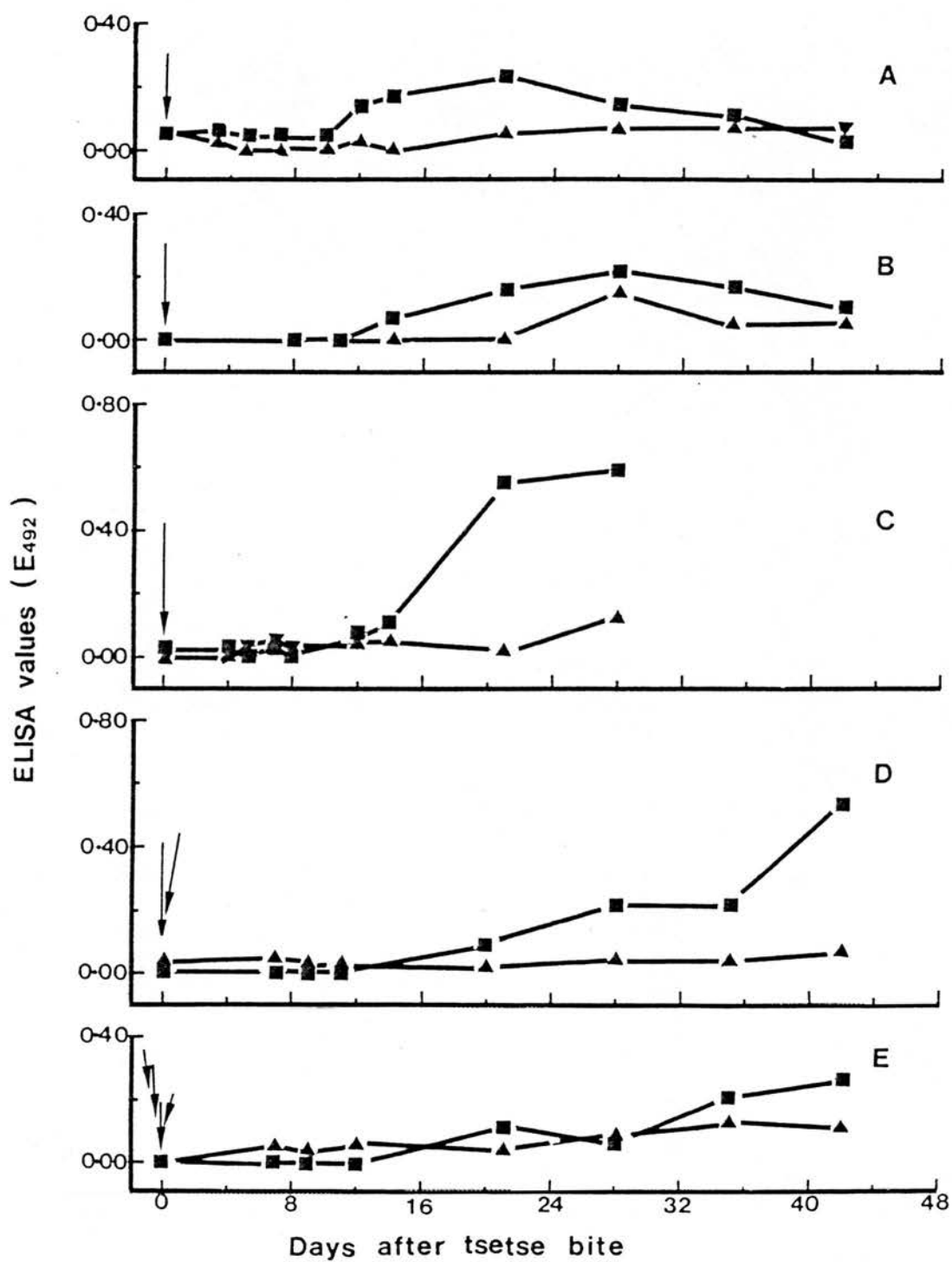




Table 6.3

Neutralization of infectivity of Trypanosoma congolense TREU 1690 using pooled sera and tissue fluid from rabbits R874 and R885.

Sample	Days After Infection									
	0	7	14	21	28	35	42	49	56	63
Number of Mice Infected/Number Inoculated										
Serum	5/5	5/5	5/5	3/5	1/5	0/5	0/5	0/5	1/5	2/5
Tissue Fluid	5/5	5/5	5/5	5/5	5/5	3/5	1/5*	1/5*	5/5*	5/5*

\*Rabbit R885 only

#### 6.4 Sterile Immunity in Rabbits after Chemotherapy of Trypanosoma congolense Infections Induced by Cultured Metacyclic Trypanosomes

Experimental design: Eighteen rabbits were each inoculated intradermally with  $5 \times 10^5$  cultured metacyclics of T. congolense TREU 1690 and then divided into two groups to be treated with 14 mg/kg diminazene aceturate at either seven or 14 days after infection. Chancre development at the time of treatment was noted and the presence of trypanosomes in the blood checked by microhaematocrit/buffy coat technique and mouse subinoculation. After treatment, each group was again randomly divided into three smaller groups of three rabbits which were challenged 28, 42 or 56 days after treatment. Three uninfected rabbits treated with 14 mg/kg diminazene aceturate and challenged at either 28, 42 or 56 days after treatment acted as controls.

Serum samples were collected at day 0 and then twice weekly up to four weeks after challenge for evaluation of anti-trypanosomal antibodies using ELISA and Neutralization of Infectivity test. All rabbits were challenged by bites from tsetse flies infected with T. congolense TREU 1690.

Results: Homologous challenge of rabbits treated at seven days after infection resulted in infections developing in all animals except rabbit 1098 challenged on day 56 (Table 6.4). Serial sera from these rabbits were negative for anti-trypanosomal antibodies by both ELISA and Neutralization of Infectivity test (Table 6.5). Serum samples from rabbit 1098 which had shown resistance on challenge were also negative for trypanosomal antibodies (Table 6.8).

In contrast, seven out of nine rabbits treated 14 days after infection showed resistance to homologous challenge (Table 6.6).

Small chancres developed in three of these immune rabbits (1095, 1103 and 1105). One rabbit from each of the groups challenged at 42 and 56 days after treatment showed development of chancres and became infected after challenge (rabbits 1099 and 1100). Control uninfected rabbits (1114, 1126 and 1127) challenged at 28, 42 and 56 days after treatment showed typical development of chancres and parasitaemia similar to untreated susceptible animals (rabbit 821, Figure 6.1C). Results of ELISA and Neutralization of Infectivity tests for the rabbits treated at 14 days after infection except 1099 and 1100, are shown in Table 6.7. ELISA values of 0.10-0.27, which would be regarded as positive for anti-trypanosomal antibodies, were obtained from serum samples collected at 14, 28, 42 and 56 days after treatment. Sera collected 28, 42 and 56 days after treatment completely neutralized the infectivity of T. congolense TREU 1725. Sera from rabbits 1099 and 1100 which were not immune, did not show anti-trypanosomal antibodies by either ELISA test or Neutralization of Infectivity test (Table 6.8).

Table 6.4

Development of infection after homologous challenge of rabbits treated with 14 mg/kg DIMINAZENE ACETURATE on day 7 after intradermal inoculation of cultured metacyclics of Trypanosoma congolense TREU 1457.

Rabbit Number	Day of Challenge Post-treatment	Number of chancres/Number of flies fed	Presence of Trypanosomes**	
			Pre-treatment	Post-challenge
1127*	28	5/6	-	+
1094	28	5/6	+	+
1106	28	3/6	0	+
1113	28	6/7	0	+
1126*	42	6/6	-	+
1097	42	6/7	+	+
1107	42	2/6	+	+
1111	42	6/6	0	+
1114*	56	5/6	-	+
1098	56	0/6	0	0
1110	56	1/6	0	+
1112	56	1/6	+	+

\*Uninfected treated controls

\*\*0 = No trypanosomes detected by mouse subinoculation

Table 6.5

Serum antibody response and immunity in nine rabbits\* infected with cultured metacyclics of Trypanosoma congolense TREU 1457 and treated on day 7 post-infection.

Test	Days Post-infection					
	0	7.	21	35	49	63
ELISA** values ( $E_{492}$ )	.01	.06	.04	.03	.09	.08
Neutralization** of infectivity:						
Number of mice uninfected/Number inoculated	1/5	1/5	0/5	0/5	0/5	0/5
Resistance to challenge:						
Number of rabbits resistant/Number challenged	-	-	-	0/3	0/3	1/3

\*As shown in Table 6.4 except controls

\*\*Tests done on pooled sera from animals before challenge, except from R1098

Table 6.6

Development of infection after homologous challenge of rabbits treated with 14 mg/kg DIMINAZENE ACETURATE on day 14 after intradermal inoculation of cultured metacyclics of Trypanosoma congolense TREU 1457.

Rabbit Number	Day of Challenge Post-treatment	Number of chancres/Number of flies fed	Presence of Trypanosomes**	
			Pre-treatment	Post-challenge
1127*	28	5/6	-	+
1095	28	4/8	+	0
1101	28	0/8	+	0
1102	28	0/8	+	0
1126*	42	6/6	-	+
1096	42	0/8	+	0
1100	42	4/6	+	+
1105	42	4/8	+	0
1114*	56	5/6	-	+
1099	56	4/6	+	+
1103	56	3/6	+	0
1104	56	0/6	0	0

\*Uninfected treated control

\*\*0 = No trypanosomes detected by mouse subinoculation

Table 6.7

Serum antibody response and immunity in nine rabbits\* infected with cultured metacyclics of Trypanosoma congolense TREU 1457 and treated on day 14 post-infection.

Test	Days Post-infection					
	0	14	28	42	56	70
ELISA** values ( $E_{492}$ )	.01	.27	.16	.11	.12	.08
Neutralization of infectivity***:						
Number of mice uninfected/Number inoculated	1/5	0/5	5/5	5/5	5/5	5/5
Resistance to challenge:						
Number of resistant rabbits/Number challenged	-	-	-	3/3	2/3	2/3

\*As shown in Table 6.6 except controls

\*\*Pooled sera from animals before challenge (Rabbits R1099 and R1100 not included)

\*\*\*Pooled sera from animals before challenge - sera from animals challenged at the same time, pooled (Rabbits R1099 and R1100 not included)

Table 6.8

Serum antibody response and immunity in rabbits R1098, R1099 and R1100 infected with cultured metacyclics of Trypanosoma congolense TREU 1457 and treated on day 7 (R1098) or day 14 post-infection.

Rabbit Number	Days Post-infection	Test		
		ELISA (E <sub>492</sub> )	Neutralization of Infectivity**	Resistance to Challenge
1098	7			
	49	.05	ND	ND
	63	.05 .07	ND 0/5	ND 0/1
1099)* 1100}	14	.16	0/5	ND
	56	.17	0/5	ND
	70	.10	0/5	0/2

\*Pooled sera

\*\*Expressed as Number of uninfected/Number of mice inoculated

ND = Not done



## 6.5 Discussion

The measurement of anti-trypanosomal antibodies by means of ELISA showed that in animals cyclically infected with T. congolense the development of serum antibodies is associated with the presence of trypanosomes in the blood. Thus, ELISA values greater than pre-infection values were detected in sera obtained from rabbits 14 or more days after infection. Following successful treatment with diminazene aceturate, values invariably decreased gradually.

In contrast, low ELISA values were found in serum samples from all rabbits in which cyclical infection was abrogated by treatment with diminazene aceturate 0-12 days post-infection, a period during which trypanosomes could not readily be detected in blood.

There were indications that animals infected with T. congolense TREU 1690 showed higher ELISA values at an earlier stage of infection than rabbits infected with T. congolense TREU 1682. It is possible that this is related to the development of higher parasitaemias at earlier stages of infection in those rabbits infected. Regular quantitation of parasitaemia in rabbits was not carried out since the numbers of organisms present were too few for estimation using either a haemocytometer or the rapid "matching" technique of Herbert and Lumsden (1976). However, it had been observed that in mice, T. congolense TREU 1690 always produced higher parasitaemias than T. congolense TREU 1682. It is also possible that the use in the ELISA test, of antigen prepared from T. congolense TREU 1690 could have contributed to higher ELISA values for sera from rabbits infected with the homologous stock of trypanosomes.

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The finding that antibody production in trypanosome infection may be related to the degree of parasitaemia has been shown to occur in other animals. In sera from cattle infected with T. brucei, peak levels of antibody assayed by Neutralization of Infectivity test, Indirect Fluorescent-antibody test (IFAT), Farr assay and Solid Radio-immunoassay (S-RIA) were shown to be proportional to the number of irradiated viable organisms inoculated intravenously (Morrison et al., 1982a).

In the present study, it was shown that in an infected untreated animal antibodies were first detectable by ELISA on day 14 post-infection. This is in agreement with the findings of Luckins and Gray (1979) who also reported that in rabbits cyclically infected with T. congolense, serum antibodies were detectable by ELISA and IFAT at 14 days post-infection. However, Morrison et al. (1982a) who inoculated cattle with irradiated T. brucei organisms intravenously, showed that peak antibody levels could be detected by Neutralization of Infectivity, IFAT, Farr Assay and S-RIA at nine or 11 days post-inoculation. While the use of more sensitive techniques and a different trypanosome species could account for this different finding, it is probable that the intravenous inoculation of large numbers of trypanosomes contributed to earlier stimulation of immunity.

An attempt to determine whether tissue fluid in the vicinity of the trypanosome chancre may contain antibodies when none could be detected in serum revealed that antibody levels in tissue fluid were always lower than serum antibody levels. In tissue fluid, ELISA values were 10-30% of those obtained for serum. This is consistent with the findings of Goodwin and Tierney (1977) who showed that in

rabbits infected with syringe-transmitted T. brucei, tissue fluid antibodies attained only 15-30% of plasma antibody levels.

The presence of low antibody levels in tissue fluid was confirmed by the Neutralization of Infectivity test. None of the tissue fluid samples could neutralize infectivity of the homologous stock of T. congolense whereas serum samples from the same animals at days 35, 42 and 49 after infection completely neutralized the homologous T. congolense suspension.

It is possible that the source of tissue fluids assayed in these experiments does not represent the body tissue fluid. However, Calnan et al. (1972) presented evidence from studies on albumin movement, protein and electrolyte levels that in rabbits, fluid within a tissue cage is not an inactive collection of isolated fluid but is representative of body interstitial fluid. Lower antibody levels in tissue fluid would be consistent with the predominance of IgM in trypanosomiasis in the early stages of infection. This class of immunoglobulin is largely confined to the bloodstream (Terry, 1976).

Nevertheless, results obtained with tissue fluid may not apply to events in the local skin reaction where large numbers of mononuclear cells including plasma cells have been observed at certain stages of the trypanosome chancre in rabbits, cattle and goats (Gray and Luckins, 1980; Emery and Moloo, 1981). Plasma cells are likely to be producing antibodies locally as trypanosome lysis and degeneration thought to be antibody-mediated, has been observed in local skin reaction to T. congolense (Luckins and Gray, 1980). Although in the present study, tsetse flies were fed on the skin directly above the tissue cage, so that local skin reactions would be directly above the tissue chamber,

it is possible that the method is not sensitive enough to detect locally produced antibodies. Attempts to detect the presence of locally produced antibodies by applying ELISA to fluid obtained after macerating trypanosome chancre at day 9 post-infection, were unsuccessful. More sensitive methods such as immunohistological techniques would be required for detection of antibody production in the chancre.

It appears that local expression of immunity is responsible for the destruction of trypanosomes deposited at the skin bite site when immune animals are challenged by infected tsetse flies. This is indicated by the differences in chancre and parasitaemia development in immune and susceptible animals. In all cases, immune animals developed only small skin reactions of 4-7 mm diameter within three days of challenge, and no trypanosomes could be detected at any time after challenge. In contrast, after challenge of non-immune rabbits, chancres appeared at tsetse bite sites and trypanosomes appeared in blood in a similar manner to that seen in primary infections.

The possibility that metacyclic trypanosomes deposited in immunised animals multiplied at the bite site and then entered the bloodstream where they were destroyed is not tenable for two reasons. Firstly, any multiplication would inevitably have led to the appearance of larger reactions. Secondly, serum antibody levels in immune rabbits were consistently low, almost at pre-infection levels at the time of challenge. A rise in antibody might be expected if trypanosomes entered the bloodstream in appreciable numbers.

It has been shown in the present work, that early trypanocidal drug treatment of cyclically transmitted T. congolense infections in

the rabbit can modify immunity against homologous challenge. Only one out of 11 rabbits treated with diminazene aceturate at 0-12 days after infection was immune to homologous challenge at 42 days after treatment. In contrast all 17 rabbits treated at 14 or more days after infection were immune to homologous challenge. This finding was corroborated by results from experiments in rabbits which had been treated with diminazene aceturate at either 7 or 14 days after intradermal inoculation of cultured metacyclics of T. congolense. Only one of nine rabbits treated at seven days after infection resisted infection upon cyclical homologous challenge by tsetse bite 4-8 weeks after treatment while seven out of nine rabbits treated at 14 days after infection were immune to similar homologous challenge.

In animals treated after infective tsetse bite or after intradermal inoculation of cultured metacyclics, immunity correlated with the presence of antibodies at the time of and soon after treatment, although antibody levels were invariably low at the time of challenge. Thus in animals which were treated 14 days after intradermal inoculation of cultured metacyclics, seven rabbits which were immune had shown evidence of trypanosome neutralizing antibodies post-treatment, whereas in two other susceptible animals which became infected following challenge, no serum neutralizing antibodies were detected. Antibodies could not be detected in any of the nine animals treated at seven days after intradermal inoculation of cultured metacyclics, although one rabbit (1098) subsequently showed resistance to homologous challenge.

The finding that immunity develops in animals treated with diminazene aceturate at 14 or more days post-infection but not in those

treated earlier is in agreement with findings from studies in cattle, in which immunity to homologous challenge was shown to develop in animals treated at 15 days post-infection, but not in those treated at five or 10 days after infection (Emery et al., 1980). In contrast, rabbits treated with homidium chloride at seven days after infection with T. congolense have been shown to be immune to homologous challenge (Luckins et al., 1983).

The different result on immunity developing after chemotherapy of cyclical T. congolense infections in rabbits as obtained by Luckins et al. (1983) compared to those obtained in the present studies is difficult to explain since the trypanosome stocks used in the two studies are related. There is a possibility that use of different trypanocidal drugs may account for the difference. Diminazene aceturate is rapid in action while homidium, like all phenanthridium drugs is slower in action allowing 3-5 cell divisions to take place after treatment (Hawking, 1958, 1963). Thus, trypanosomes in a homidium-treated animal would be available in larger numbers and for a longer time after treatment than in an animal similarly treated with diminazene aceturate.

Other studies have shown that rabbits treated with homidium chloride at seven days after intradermal inoculation of cultured metacyclics of T. congolense are not immune to challenge at 28-56 days after treatment in contrast to rabbits similarly challenged after treatment at 14 days after infection (Luckins, unpublished).

Some findings on immunity developing in mice treated with diminazene aceturate after cyclical T. congolense infection, support the findings from the present studies in rabbits. Nantulya et al. (1980b) reported that mice given a single course of 5-12 bites from T. congolense

infected flies and then treated with diminazene aceturate at 10 days after first infectious bite, were not immune to homologous challenge one week later. In contrast, mice given two courses of infection and treatment separated by three-week intervals were immune to homologous challenge. Since patent parasitaemia in control mice was not established until 10-12 days after infection, it is possible that when treatment was carried out at 10 days post-infection, parasitaemia had not reached the requisite level for induction of comprehensive immunity. Mice treated with diminazene aceturate at seven days after a single intraperitoneal inoculation of  $10^6$  cultured metacyclics of T. congolense have been shown to be immune to homologous challenge even though no parasitaemia could be detected at the time of treatment (Varma, 1983). It has also been shown that mice infected with blood-stream forms of T. brucei and then treated with diminazene aceturate at seven days post-infection when they were parasitaemic, resisted homologous challenge 42-370 days after treatment (Herbert and Lumsden, 1968).

While the results presented above showed that rabbits treated at < 14 days after cyclical infection are not immune to homologous challenge, limited observations show that two courses of infection and treatment at less than 14 days post-infection may lead to the development of sterile immunity. Two rabbits which had been infected by bites from five infected flies each, on two consecutive occasions, each followed by treatment at less than seven days after tsetse bite, were shown to be immune to homologous challenge 40-42 days after the second treatment (Luckins, Silayo and Rae, 1982). This is in agreement with the findings of Nantulya et al. (1980b) who reported that mice were

immune after two consecutive infections and treatments when single infection and treatment did not lead to the development of immunity. There is however, a need for further studies to confirm the above observations. If such development of immunity after two consecutive infections and treatments also occurs in cattle, it is difficult to explain why Wilson et al. (1975, 1976) failed to observe the development of immunity in animals treated with diminazene aceturate as a group whenever one individual was positive for trypanosomiasis. Since Wilson et al. (1975, 1976) found that animals treated only after they were positive for infection acquired immunity, a plausible explanation based on the findings of Morrison et al. (1982a) in cattle and from the present studies in rabbits is that most of the animals treated as a group whenever one animal was found infected, were treated at too early a stage of infection and a comprehensive immunity against the infecting stock of trypanosomes was unable to develop.



## CHAPTER SEVEN

## GENERAL DISCUSSION

The present studies in rabbits have shown that the local skin reaction or chancre, in which T. congolense initially develops extra-vascularly after infective tsetse bite, may influence the efficacy of chemotherapy and subsequent development of sterile immunity. In addition it was shown that early chemotherapy abrogates further development of the trypanosome chancre.

It would appear that one of the reasons why trypanosome chancres have not been reported to occur in cattle under natural field conditions is mass application of chemotherapy. In the present studies it was shown that rabbits treated with diminazene aceturate up to three days after infective tsetse bite did not develop chancres. When treatment was carried out 4-7 days after the infective tsetse bite, the effect was to reduce the size of the chancre compared to those on normal untreated animals at the same period after treatment. Animals treated at eight or more days after infective tsetse bite had chancres which did not differ appreciably from those on normal untreated rabbits. Histological examination of chancres after treatment revealed that, although trypanosomes could not be detected 2-4 days after treatment, the cellular reaction in composition and extent was similar to that in untreated animals at the same time after infection.

It is not clear why the trypanosome chancre in untreated animals disappears at 14-18 days after infection. It has been suggested that in the case of the T. congolense chancre, this may be related to a biological change associated with maturation of inoculated metacyclic

trypanosomes into bloodstream forms (Akol and Murray, 1982). However this would be incompatible with the observation that bloodstream forms of T. brucei when inoculated intradermally, also elicit chancre formation (Willett, 1956; Awad et al., 1969; Emery et al., 1980).

It is suggested that the chancre in untreated rabbits disappears at 14-18 days post-infection because of appearance of serum antibodies at 14 days after infection. This theory would be compatible with previous suggestions that the chancre is a type of Arthus reaction (de Raadt, 1974) and that immune complex reactions are most severe when antigen is in excess (Herbert and Parratt, 1979). Thus the effect of increasing antibody production would be destruction of trypanosomes and a change in the ratio of antigen to antibody, eventually leading to disappearance of the chancre. Some observations on chancre development in animals treated with isometamidium chloride would tend to support this theory. It was observed in the present studies that in some rabbits treated with isometamidium chloride and then cyclically challenged with T. congolense, chancres which appeared, remained up to 50 days after challenge. Although serum antibody levels in these rabbits were not determined, it is possible that residual drug in plasma would initially have prevented establishment of parasitaemia essential for initiation of antibody production.

The view that the trypanosome chancre is a type of Arthus reaction is supported by the finding that an Arthus type reaction can be elicited in infected rabbits or infected treated rabbits by intradermal inoculation of sonicated T. congolense antigen, and in normal rabbits by intradermal inoculation of antigen-antibody complex (Mansfield and

Kreier, 1972b). Further support comes from the observation that chancre size is proportional to the number of metacyclics inoculated intradermally (Luckins et al., 1981). Argument that such a proportionality could be due to a phospholipase activity resulting from trypanolysis in tissues (Losos and Chouinard, 1979) is not tenable because if this were the case it would be expected that the appearance of antibodies at 14-18 days post-infection would result in an increase in the reaction size as more trypanosomes are destroyed 14 days post-infection.

Argument against ascribing the chancre to Arthus-type reaction solely, is that in the classical Arthus reaction (Ranadive and Mowat, 1979) antibody and antigen occur on opposite sides of the vascular wall. This is clearly not the case at days 6-14 post-infection since antibody levels in serum were low at that stage. Thus any antibody involved in immune complex formation would be in the same tissues in which the antigen is present. It may therefore be that the chancre is characterised by an initial weak Arthus reaction in which the presence of trypanosomes in collagen, leads to infiltration with polymorphonuclear cells, vasodilation and mononuclear cell infiltration. Thus the chancre may be taken as both an immediate hypersensitivity and inflammatory reaction. The role of delayed hypersensitivity reaction also needs to be investigated since it has been shown that a delayed-type skin reaction can be elicited in T. congolense-infected cattle inoculated with ultrasonicated or formalin fixed T. congolense (Emery, Wells and Tenywa, 1980).

The effectiveness of chemotherapy appears to depend on the stage of development of T. congolense chancres at the time at which

trypanocidal drug is administered. Thus a high incidence of relapse parasitaemias developed when treatment was carried out at 8-10 days after infection when chemotherapy was found to be ineffective in 50% of the treated animals. Even when the dose of diminazene aceturate was increased to 14 mg/kg one out of six treated rabbits had relapse parasitaemia. In contrast, 29 rabbits treated with 7 mg/kg diminazene aceturate at 0-4 or 12-42 days were completely cured.

A finding of considerable importance in relation to chemotherapy was the observation that extravascular foci of trypanosomes might be responsible for relapses in parasitaemia following treatment of drug sensitive stocks of T. congolense with therapeutic doses of diminazene aceturate. Treatment carried out 8-10 days post-infection probably precludes the involvement of parasites in the general circulation as a source of relapses, since firstly the numbers present are low and secondly, treatments carried out much later when systemic infections have been fully established have been shown to be effective. Thus, under certain conditions, the extravascular foci of T. congolense development in the chancre might present a source of trypanosomes which have evaded the effects of the drug. It is suggested that relapses occur due to drug concentration in tissue fluid being insufficient for the high concentration of trypanosomes in some chancres at 8-10 days post-infection. There have been previous studies which indicated that the efficacy of trypanocidal drug treatment may be affected by concentration of trypanosomes at the time of treatment (Reviewed by Davey, 1957; Hawking, 1963a). More recently, it has been shown that in mice the minimum curative dose of diminazene aceturate for T. congolense infection is proportional to the infecting dose of trypanosomes (Raether, 1970; Walker and Opiyo, 1973).

A number of relapse infections were accompanied by the development of secondary chancres. Histological studies of skin sections from chancres removed at 29 and 33 days post-infection in two rabbits showing such secondary chancres failed to reveal the presence of trypanosomes but the cellular reaction observed consisted of massive proliferation of plasma cells. There is clearly a need to carry out more studies on this form of drug failure particularly with cattle as the experimental animals to determine the importance and pathogenesis of the secondary chancres.

The finding that rabbits were always cured when treated at 12 days or more after infection contrasts with recent findings that in mice infected with T. brucei, relapse infections attributable to brain inhabiting trypanosomes occurred when treatment with diminazene aceturate was carried out at 14 days or more after infection (Jennings et al., 1977; 1979; 1980). It is not clear whether such relapse infections occur in natural infections in cattle although there have been reports of T. congolense isolated from the brain but not from the organs or blood of an ox treated with diminazene acetate after triple infection with T. brucei, T. congolense and T. vivax (Haase, Bernard and Guidot, 1981).

Diminazene acetate has little prophylactic effect against T. congolense cyclically transmitted by tsetse-fly or by intradermal inoculation of cultured metacyclics. When metacyclics of T. congolense were inoculated intravenously, it was shown that the drug has a prophylactic effect of up to 24 hours only. Similar findings have been reported by other workers using mice and rabbits (Frank and Dann, 1974; Nantulya et al., 1980b; Whitelaw, 1982; Gilbert, 1983). This is in

contrast to reports that in cattle challenged with T. congolense bloodstream forms inoculated intravenously, diminazene aceturate exerts a prophylactic effect of up to 12 days after treatment (Wellde and Chumo, 1983). The discrepancy could be due to host species differences, as well as use of different trypanosome stocks and inoculum doses. Wellde and Chumo (1983) did not state whether absence of parasitaemia was confirmed by subinoculation of blood into mice as was carried out in the present studies. It is likely that parasitaemias in animals in which residual levels of trypanocidal drugs were present would be low and not easily detected by conventional microscopy.

The lack of prophylactic activity of diminazene aceturate against T. congolense in mice and rabbits contrasts with reported prolonged prophylactic effect against T. brucei in mice, rats and monkeys (Lumsden et al., 1965; Raether et al., 1972; 1974; Fink and Dann, 1974; Zahalsky and Weinberg, 1976; Whitelaw, 1982). This is possibly due to different actions of the drug on the two trypanosome species since it has been shown that higher doses of diminazene aceturate are required to abolish infectivity of T. congolense compared to T. brucei (James, 1976).

Isometamidium chloride at 4 mg/kg dose rate in the rabbit has a prophylactic effect of less than three weeks against cyclically transmitted T. congolense. This drug dose was calculated to be equivalent to a dose of 1 mg/kg used for prophylaxis in cattle. It had been shown that 0.75 mg/kg isometamidium chloride is curative for established infections of T. congolense TREU 1690 in the rabbit. The result is therefore in contrast to reports from field studies which

showed that 1-2 mg/kg isometamidium chloride has a prophylactic effect which persists for 10-13 weeks (Robson, 1962; Kirkby, 1964; Wiesenhutter, Turner and Kristensen, 1968). It is not clear whether this discrepancy is due to host-species variation or due to differing diagnostic methods. In the field trials the methods used for detecting trypanosomes do not appear to have included subinoculation of blood into mice.

An interesting finding from studies on prophylactic effect of isometamidium chloride and diminazene aceturate against cyclically transmitted T. congolense was that chancres sometimes appeared without subsequent systemic establishment of infections. This would indicate that drug levels in serum but not in tissue fluid were sufficient to prevent infection. However, it has been shown that three hours to seven days after treatment of rabbits with radio-labelled diminazene aceturate tissue fluid contains higher residue drug levels than plasma (Gilbert and Newton, 1982b; Gilbert, 1983). No similar radio-isotope studies have been carried out on isometamidium chloride but Goodwin and Tierney (1977), using a biological assay technique, showed that isometamidium levels which were detectable in tissue fluid and plasma for only one week after intramuscular injection of 10 mg/kg dose rate, were higher in tissue fluid than plasma. More recently, using a chemical assay technique, Braide and Eghianruwa (1980) were able to detect isometamidium chloride in serum at 24 hours but not seven or more days after intramuscular injection of therapeutic dose of 0.5 mg/kg in goats.

It is possible that transient parasitaemia occurring after challenge of drug treated animals could have occurred but remain undetected by microhaematocrit/buffy coat technique and mouse subinoculation.



This problem has been highlighted by Gray, Jennings and Hajduk (1982) who showed that in mice, following diminazene treatment of pleomorphic T. brucei infections initiated with fly-derived metacyclics, early transient relapses occurred which were characterised by low numbers of uninformative trypanosomes. It would be interesting to determine whether such transient infections occur in animals challenged while under chemoprophylaxis and whether sterile immunity can subsequently be demonstrated. Preliminary results (not included here) from application of ELISA test on a few serial serum samples from some of the isometamidium treated rabbits which developed chancres but not parasitaemia indicate that these animals do not develop anti-trypanosome antibodies.

In the present studies, it was shown that rabbits treated with diminazene aceturate at up to 12 days after cyclical infection were found to be susceptible to homologous challenge while those treated at 14 or more days after infection were immune. This result was corroborated by findings from studies in which rabbits were infected by intradermal inoculation of large numbers of cultured metacyclic forms of T. congolense and treated with diminazene aceturate at seven or 14 days post-infection. Sterile immunity to homologous challenge by tsetse bite 28-56 days after treatment was demonstrated only in rabbits treated at 14 days post-infection. This result is in agreement with findings in cattle, in which it was shown that treatment with diminazene aceturate at 15 days post-infection with cyclically transmitted T. congolense conferred sterile immunity, while similar treatment at five or 10 days post-infection did not confer immunity (Emery et al., 1980).



In contrast, rabbits treated with homidium chloride seven days after cyclical infection with T. congolense were shown to be immune to homologous challenge up to 300 days post treatment (Luckins et al., 1983).

The discrepancy between results obtained in rabbits treated with diminazene aceturate in the present studies and those obtained by Luckins et al. (1983) in rabbits treated with homidium chloride is difficult to explain. In the present studies it was shown that acquisition of immunity was associated with the presence of trypanocidal antibodies detectable by ELISA at treatment but not necessarily at the time of challenge. Luckins et al. (1983) also reported that at the time of challenge, immune animals did not necessarily have detectable antibody levels in serum.

The acquisition of immunity in animals treated with diminazene aceturate at 14 days post-infection but not in those treated at seven day post-infection can be related to differences in level of parasitaemia at these two different times after infection. In the present studies, it was found that parasitaemia in cyclically infected rabbits could be detected by microhaematocrit/buffy coat technique at 12-14 days but usually not before. Antibody was also first detected at 14 days post-infection. It can thus be argued that for development of sterile immunity to T. congolense a certain minimal blood parasitaemia is required. This suggestion is supported by studies in cattle immunised with irradiated T. brucei organisms (Morrison et al., 1982a). Animals immunised by inoculation of  $10^7$  or more irradiated T. brucei organisms intravenously were immune to challenge with  $10^3$  homologous trypanosomes while those inoculated with  $10^6$  organisms or less were

not completely immune. The duration of time during which the inoculated irradiated trypanosomes circulated was not determined but is most likely to have been less than seven days. Thus it is possible to immunise animals by infection and treatment if a high enough parasitaemia is achieved before treatment.

It is possible that the differences in acquisition of immunity, in rabbits treated with either diminazene aceturate or homidium chloride is due to differences in drug action. Diminazene aceturate is known to act rapidly while homidium chloride acts slowly, allowing 2-3 cell divisions to take place after treatment. Thus in the homidium treated animals antigen is available for a longer time and in larger amounts than similarly infected animals treated with diminazene aceturate at the same time.

If as suggested above, immunity develops only when a certain blood parasitaemia is attained, it is still not clear whether this requisite level of parasitaemia provides an essential antigen mass or the essential VAT composition for development of comprehensive serodeme-specific immunity. Most likely it is a combination of both. It is interesting to speculate on why in spite of there being a trypanosome population exceeding  $10^7$  in the chancre at peak chancre size stage, 8-13 days post-infection (Willett and Gordon, 1957; Emery and Moloo, 1981) no comprehensive immunity has developed by day 12 post-infection. It could be that in the chancre only limited number of VATs are expressed. It may also be that limited antigen processing takes place in the chancre and the regional lymph node up to 14 days post-infection due to inaccessibility of trypanosome antigen within the collagen in spite of the massive cellular reaction seen in the

chancre. However the situation may be more complicated than that, in view of the finding that homidium treated animals develop comprehensive immunity when treatment is carried out at seven days post-infection (Luckins et al., 1981).

Since the initial VATs to which the animal is exposed after cyclical challenge are the metacyclic VATs, comprehensive sterile immunity against particular serodemes must include immunity against all the metacyclic VATs. Recent studies have shown that the VAT repertoire of metacyclic forms of T. congolense TREU 1457 (from which T. congolense TREU 1690 used in the immunity studies was derived) is limited to 12 VATs (Crowe et al., 1983). It is possible, therefore, that within 14 days of infection by tsetse-fly bite or intradermal inoculation of cultured metacyclic trypanosomes, the immune system has been exposed to trypanosomes of 12 different metacyclic VATs either concurrently or consecutively, in sufficient numbers for immune stimulation. However it may also be that not all the metacyclic VAT repertoire need be expressed for stimulation of serodeme specific immunity. There is thus a clear need for more studies to determine what proportion or which sequence of VAT repertoire need develop systemically for serodeme-specific immunity to develop.

After homologous challenge of immune animals with tsetse flies no trypanosome chancres developed at the sites of tsetse bites although in some rabbits small reactions were observed to develop 2-5 days post-infection. No attempt was made to determine whether these small reactions contained trypanosomes, but they were most likely similar to reactions observed in cattle and goats even at sites where non-infected flies had fed on animals previously bitten by tsetse flies (Emery et al.,

1980; Akol and Murray, 1983) and which resembled typical Arthus reactions. This lack of typical chancre development would indicate that immunity is elicited at the skin level, against the metacyclic VATs.

Studies in cattle have shown that immunity induced by infection and treatment is not dependent on the VAT composition of the infective blood meal offered to the flies used for primary or subsequent challenge infection (Akol and Murray, 1983). This was also the case in the present work; trypanosomes used for initiating infections in flies originated from different stabilates and different passages of the same trypanosome stock and infections in host mice were of varying duration (2-8 days). However no attempt was made to determine the degree of homogeneity or otherwise of the trypanosomes in different infective feeds.

The finding that animals immunised with cultured metacyclics were also immune to challenge with tsetse transmitted metacyclics points to the potential use of such antigens for immunisation programmes. Further studies would have to be carried out to determine how many different serodemes could be inoculated simultaneously and yet lead to development of sterile immunity against all inoculated stocks.

The present studies indicate that, failure of chemotherapy regimes to engender development of sterile immunity in the field may be due to treatments carried out too soon after infection thus preventing development of immunity as was seen in animals treated at less than 14 days post-infection. The findings from the field studies of Wilson et al. (1975; 1976) can be interpreted in this way. Animals

which were treated with diminazene aceturate only when they developed infection, acquired immunity in contrast to those treated as a group whenever one individual in the group was infected. Hence attempts to develop a more rational approach to chemotherapy taking account of tsetse challenge and frequency of infection may enhance protective immunity in herds at risk.

It is clear that there are many aspects of development of immunity in relation to chemotherapy of cyclically transmitted infections which should have been studied. Unfortunately the experiments on development of sterile immunity were of long duration, occasioned by an interval of six weeks between treatment and challenge of animals, to allow relapse infections to be detected. Thus limited time and animal housing space, precluded more in-depth studies.

Nevertheless the studies have been useful in pointing out the significance of the trypanosome chancres on efficacy of chemotherapy and in showing that diminazene aceturate has little prophylactic activity against cyclically transmitted T. congolense in rabbits. Since the results were in agreement with those obtained in T. congolense-infected cattle treated with diminazene aceturate, the rabbit could be a suitable cheap model laboratory animal for studies on cyclically transmitted T. congolense infections. The serodeme-specificity of the chancre (Emery et al., 1980; Luckins et al., 1983; Luckins and Gray, 1983; Akol and Murray, 1983) is allowing serodeme identification by chancre induction (ILRAD, 1983). The rabbit would be particularly useful for this purpose.

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APPENDIX 1FORMULAE FOR PREPARING BUFFER SOLUTIONS IN ONE LITRE AMOUNTS1. Phosphate Buffered Saline Glucose (PBSG), pH 8.0

di-sodium hydrogen orthophosphate, anhydrous ( $\text{Na}_2\text{HPO}_4$ )	8.094 g
sodium di-hydrogen orthophosphate $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	0.468 g
Sodium chloride ( $\text{NaCl}$ )	2.55 g
Distilled water	up to 1000 ml
Glucose	1% in PBS

2. Bicarbonate/carbonate Buffer pH 9.6

(ELISA antigen-coating buffer)

Sodium carbonate ( $\text{Na}_2\text{CO}_3$ )	1.59 g
Sodium hydrogen carbonate ( $\text{NaHCO}_3$ )	2.93 g
Sodium azide ( $\text{NaN}_3$ )	0.2 g
Distilled water	up to 1000 ml

3. Phosphate Buffered Saline (PBS) pH 7.4

Potassium chloride $\text{KCl}$	0.2 g
Potassium di-hydrogen orthophosphate ( $\text{KH}_2\text{PO}_4$ )	8.0 g
di-sodium hydrogen phosphate dodecahydrate $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$	2.89 g
Distilled water	up to 1000 ml

4. Phosphate Citrate Buffer pH 6.0

0.1M Citric acid	368.5 ml
0.2M di-sodium hydrogen orthophosphate ( $\text{Na}_2\text{HPO}_4$ )	631.5 ml



APPENDIX 2Giemsa-collophonium staining technique

1. Place sections in La Manna's\* Fluid and leave overnight.
2. Stain in 3% Giemsa stain overnight.
3. Differentiate in collophonium (1% in 95% Methyl alcohol) observing under the microscope until desired degree of differentiation has been achieved.
4. Dehydrate in isopropyl alcohol, clean and mount.

\*La Manna's Fluid

Potassium dichromate	9.5 g
Zinc chloride	4.5 g
Distilled water	to 100 ml



APPENDIX 3  
Packed cell volumes (PCV) of T. congolense-infected and non-infected rabbits

Rabbit Group	Weeks Post-infection											
	-1	0	1	2	3	4	5	6	7	8	9	10
Packed Cell Volume $\pm$ SD												
Infected*												
Treated (n = 4)	39.5 $\pm$ 1.0	39.3 $\pm$ 1.3	36.3 $\pm$ 3.5	32.8 $\pm$ 2.1	34.0 $\pm$ 2.3	34.5 $\pm$ 1.9	32.0 $\pm$ 2.8	29.5 $\pm$ 2.6	32.8 $\pm$ 4.8	40.5 $\pm$ 1.3	42.5 $\pm$ 2.4	42.8 $\pm$ 4.2
Uninfected**												
Treated (n = 3)	43.3 $\pm$ 2.1	40.3 $\pm$ 2.3	38.3 $\pm$ 0.6	38.0 $\pm$ 2.6	40.0 $\pm$ 0.0	41.3 $\pm$ 1.5	41.0 $\pm$ 1.0	42.0 $\pm$ 1.0	43.7 $\pm$ 2.1	42.7 $\pm$ 2.3	42.3 $\pm$ 3.1	42.7 $\pm$ 2.9
Uninfected***												
Untreated (n = 3)	40.0 $\pm$ 1.0	38.3 $\pm$ 1.5	39.3 $\pm$ 0.6	39.7 $\pm$ 1.2	39.3 $\pm$ 1.2	41.0 $\pm$ 1.0	40.7 $\pm$ 0.6	41.0 $\pm$ 1.7	41.3 $\pm$ 1.5	42.0 $\pm$ 2.0	42.3 $\pm$ 1.5	42.3 $\pm$ 1.5

\*Rabbits R1069, R1073, R1075 and R1077

\*\*Rabbits R1067, R1070 and R1071

\*\*\*Rabbits R1072, R1074 and R1076

## APPENDIX 4

Mean weights (kg) of T. congolense-infected and non-infected rabbits.

Rabbit Group	Weeks Post-infection											
	0	1	2	3	4	5	6	7	8	9	10	12
	Weight Kg $\pm$ SD											
Infected*												
Treated (n = 4)	2.35 $\pm$ .21	2.55 $\pm$ .29	2.83 $\pm$ .32	2.97 $\pm$ .28	3.10 $\pm$ .28	3.16 $\pm$ .23	3.25 $\pm$ .23	3.20 $\pm$ .31	3.36 $\pm$ .26	3.41 $\pm$ .23	3.46 $\pm$ .20	3.70 $\pm$ .36
Uninfected**												
Treated (n = 3)	2.07 $\pm$ .04	2.40 $\pm$ .29	2.74 $\pm$ .19	2.94 $\pm$ .19	3.11 $\pm$ .15	3.29 $\pm$ .15	3.33 $\pm$ .20	3.40 $\pm$ .16	3.42 $\pm$ .25	3.44 $\pm$ .39	3.48 $\pm$ .24	3.57 $\pm$ .29
Uninfected***												
Untreated (n = 3)	2.23 $\pm$ .04	2.61 $\pm$ .07	2.81 $\pm$ .10	2.90 $\pm$ .10	3.00 $\pm$ .16	3.03 $\pm$ .13	3.10 $\pm$ .14	3.10 $\pm$ .13	3.08 $\pm$ .14	3.04 $\pm$ .16	3.06 $\pm$ .18	3.38 $\pm$ .22

\*Rabbits R1069, R1073, R1075 and R1077

\*\*Rabbits R1067, R1070 and R1071

\*\*\*Rabbits R1072, R1074 and R1076